Biosci. Biotechnol. Biochem., **73** (2), 311–315, 2009

Pro-Senescent Effect of Hydrogen Peroxide on Cancer Cells and Its Possible Application to Tumor Suppression

Kaichi YOSHIZAKI, Tsukasa FUJIKI, Takahiro TSUNEMATSU, Makiko YAMASHITA, Miyako UDONO, Sanetaka SHIRAHATA, and Yoshinori KATAKURA†

Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

Received July 29, 2008; Accepted September 30, 2008; Online Publication, February 7, 2009
[doi:10.1271/bbb.80517]

Mild oxidative stress is known to induce premature senescence, termed stress-induced premature senescence (SIPS), in normal human diploid cells. We investigated to determine whether mild oxidative stress would trigger SIPS in a human tumor cell line, human lung adenocarcinoma A549. The results showed that sublethal concentrations of H_2O_2 induced SIPS in A549 cells and consequently attenuated, but did not completely eliminate, the tumorigenicity of these cells. We next investigated the reasons for this incomplete impairment of tumorigenicity in A549 cells in SIPS. The results suggested that H_2O_2-treated A549 cells are composed of a heterogeneous cell population: one is sensitive to H_2O_2, and the other is resistant or undergoes reversal; the latter reverted to their original tumorigenic form. The molecular mechanisms determining the cellular fate of tumor cells in SIPS should be identified in order to make use of SIPS and oncogene-induced senescence in tumor cells as methods of tumor suppression.

Key words: premature senescence; oxidative stress; hydrogen peroxide; A549; tumorigenicity

Various human proliferative cells exposed *in vitro* to many types of subcytotoxic stresses, including H_2O_2, hyperoxia, and UV radiation, develop stress-induced premature senescence (SIPS). Among these stresses, SIPS induced by sublethal treatment with H_2O_2 in human diploid fibroblasts has been extensively studied.[1] Researchers have found that this type of SIPS is induced independently of telomerase,[2] and that cells in SIPS exhibit cellular senescence markers, including senescent-morphological features, irreversible G1 cell cycle arrest, irreversible senescence-associated β-galactosidase (SA-β-Gal) activity, increased expression of cell cycle marker genes, and differential expression of MAPK p38-target genes,[3,4] which is likely to indicate cells in replicative senescence.[5,6]

We have found that transforming growth factor-β1 (TGF-β1), which is known to regulate the induction of several senescence markers in SIPS,[7] induces a cellular senescence-like state in human tumor cells.[8] Although it is known that several treatments, including chemotherapeutic drugs, radiation, and differentiation agents, can induce SIPS in tumor cells, it has not yet been established whether sublethal treatment with H_2O_2 can induce SIPS in human tumor cells as well as in normal ones. Hence, in this study, we first attempted to clarify the SIPS-inducing ability of sublethal H_2O_2 treatment of tumor cells.

Recently, senescence induced by oncogenes was found to reflect a natural surveillance mechanism embedded in cellular proliferative programs that protects against tumorigenesis.[9–12] In this study, we attempted to detect changes in the tumorigenicity of tumor cells upon induction of SIPS by treatment with sublethal concentrations of H_2O_2.

Materials and Methods

Cell culture. A549 cells (JCRB0076; HSRRB, Osaka, Japan) and its sublines were cultured in eRDF medium (Kyokuto Pharmaceutical, Tokyo) supplemented with 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) at 37 °C in 5% CO_2. TIG-1 cells (Institute of Development, Aging, and Cancer, Tohoku University, Miyagi) were cultured in MEM medium (Nissui, Tokyo, Japan) supplemented with 10% FBS at 37 °C in 5% CO_2. The cells were treated with 100 μM H_2O_2 for 2 h and subsequently cultured in fresh medium.

SA-β-Gal assay. SA-β-Gal assay was performed according to the method described by Dimri *et al.*[13] Staining was carried out at 37 °C for 12 h.

Immunoblot analysis. Cells were lysed in NP-40 lysis buffer (0.5% NP-40, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 1 mM PMSF, and 5 μg/ml of aprotinin). Total protein (30 μg) was subjected to 10% SDS–PAGE and transferred to a PVDF membrane (Hybond-P; GE Healthcare, Buckinghamshire, UK). The membrane was then blocked with 5% skim milk/12 mM sodium phosphate, pH 7.2, 2.7 mM KCl, and 0.14 M NaCl solution (PBS) containing 0.1% Tween (T-PBS), followed by incubation with anti-p53 (Calbiochem, San Diego, CA), anti-p21 (Santa Cruz Biotech., Santa Cruz, CA), or anti-Rb (Cell Signaling, Beverly, MA). After incubation with sheep anti-mouse IgG-HRP conjugate (GE Healthcare), the proteins were immunodetected using the ECL Plus Western Blotting Detection System (GE Healthcare).

Colony-formation assay. Colony-formation assay was performed in soft agar. Cells (1×10^4) suspended in culture medium containing 0.3% agar were seeded onto the top agar plate containing 0.6% agar, cells were cultured at 37 °C for 12–15 d, and the number of visible colonies was counted.

Tumorigenicity assay. Four-week-old nude female mice (BALB/c nu/nu; Charles River, Wilmington, MA) were administered subcutaneous injections of 1×10^7 A549 cells, A549 cells treated with H_2O_2,

† To whom correspondence should be addressed. Tel/Fax: +81-92-642-3050; E-mail: katakura@grt.kyushu-u.ac.jp

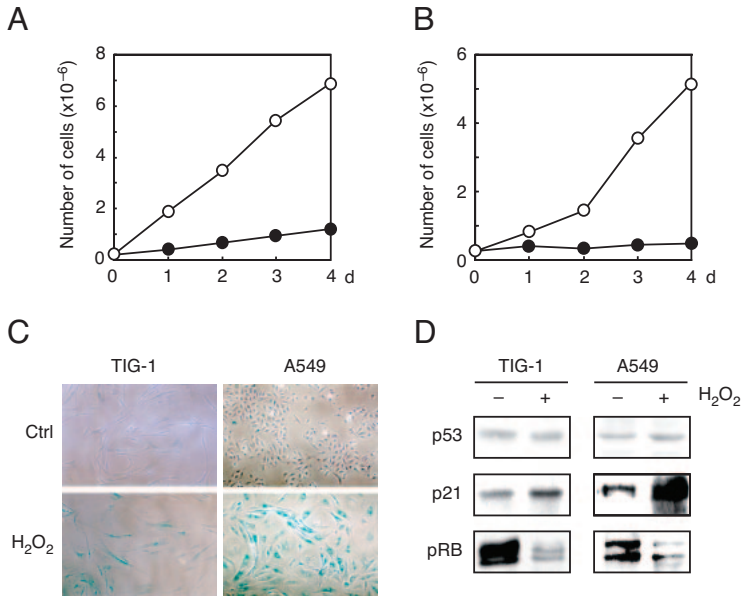

Fig. 1. Mild Oxidative Stress Triggered Premature Senescence in Tumor Cells.
Growth curves for TIG-1 cells (A) and A549 cells (B) that were (●) or were not (○) exposed to 100 μM H_2O_2 for 2 h. C, SA-β-Gal activity in TIG-1 cells and in A549 cells that were or were not treated with 100 μM H_2O_2 for 2 h. D, Total amounts of the indicated proteins were determined by western blotting of lysates from H_2O_2-treated TIG-1 and A549 cells.

and its sublines. Fifteen to 17 d after injection, tumor size was measured with a caliper and the tumor area was calculated. Representative results are shown (***, $>1.0\,cm^2$; **, $>0.5\,cm^2$; *, $<0.5\,cm^2$). Statistical difference was determined by two-sided Student's t test or Tukey's all-pairwise comparison test. This experiment was carried out following the "Guidance for Animal Experiments of the Faculty of Agriculture and the Graduate Program of Kyushu University" and Law No. 105 and Notification No. 6 of the Government of Japan.

Results

Mild oxidative stress triggered premature senescence in tumor cells

Mild oxidative stress induces cellular senescence in human diploid fibroblasts, which is termed stress-induced premature senescence (SIPS). We tested the ability of sublethal concentrations of H_2O_2 to trigger premature senescence in tumor cells as well as in normal diploid cells. As shown in Fig. 1A, B, and C, growth arrest, morphological change into an enlarged and flattened shape, and SA-β-Gal activity were induced by treatment with 100 μM H_2O_2 for 2 h in human adenocarcinoma A549 cells as well as in normal human diploid fibroblast TIG-1 cells. Further, TIG-1 cells and A549 cells exposed to mild oxidative stress showed augmented p21 expression and relatively decreased hyperphosphorylated pRb, although significant changes in p53 expression were not observed under the H_2O_2 treatment in either type of cell (Fig. 1D). All these indicate showed that H_2O_2 induced cellular senescence in human tumor cells as well as in normal human cells.

H_2O_2 treatment attenuated tumorigenicity of tumor cells

We tested the tumorigenicity of A549 cells exposed to sublethal concentrations of H_2O_2. First we evaluated the *in vitro* tumorigenicity of H_2O_2-exposed A549 cells by colony-formation assay on a soft-agar plate. After A549 cells were exposed to 100 μM H_2O_2 for 2 h, the cells were seeded onto a soft-agar plate and cultured for 12–15 d. As shown in Fig. 2A, the number and size of colonies of A549 cells formed on the soft-agar plate decreased upon treatment with H_2O_2, suggesting that H_2O_2 attenuated the *in vitro* tumorigenicity of the A549 cells.

Next we evaluated the *in vivo* tumorigenicity of A549 cells exposed to H_2O_2 in nude mice. After treatment of A549 cells with 100 μM H_2O_2 for 2 h, the cells were subcutaneously injected into the backs of the mice. A total of 15 d after injection, tumor size was measured. The results indicated that tumor formation was strongly suppressed in mice injected with A549 cells treated with H_2O_2. However, although the tumor size was considerably smaller than in the control mice, measurable tumors formed in the mice injected with A549 cells exposed to H_2O_2 (Fig. 2B). These results suggest that the tumorigenicity of A549 cells was attenuated, but not completely lost, upon treatment with H_2O_2.

Tumorigenicity of A549 cells treated with H_2O_2

We further investigated the tumorigenicity of A549 cells treated with H_2O_2. First, A549 cells were treated with 100 μM H_2O_2 for 2 h, seeded onto soft-agar plates, and cultured in the absence of H_2O_2 for 36 d. The number of visible colonies was counted at the times indicated. The results indicated that the number of colonies remained nearly constant during culture (Fig. 3A). We then investigated the effect of recovery culture of H_2O_2-treated A549 cells for the indicated periods in the absence of H_2O_2 on colony-formation ability. The results indicated that the number of colonies increased with the period of recovery culture (Fig. 3B). All these results suggest that H_2O_2-treated A549 cells are composed of a heterogeneous cell population, and that they contain cells resistant to H_2O_2, and/or that the senescence phenotypes induced by H_2O_2 in A549 cells are reversible. As Fig. 3B shows, some H_2O_2-treated

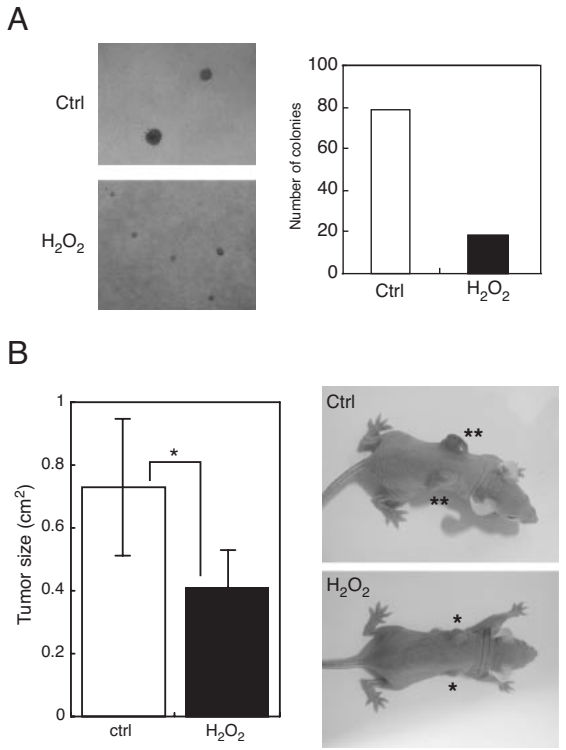

Fig. 2. H_2O_2 Treatment Attenuated Tumorigenicity of Cancer Cells.
A, Non-treated and H_2O_2-treated A549 cells (1×10^4 cells) were seeded onto a culture medium containing 0.3% agar and cultured at 37 °C for 12–15 d. The number of visible colonies was counted. Independent experiments were done 3 times, and representative results are shown. B, Non-treated and H_2O_2-treated A549 cells (1×10^7 cells) were injected subcutaneously into the backs of 4-week-old nude mice (n = 6). Fifteen days after injection, the tumor size was measured with a caliper, and its area was calculated. Statistical difference was determined by two-sided Student's t test. Differences at $P < 0.05$ were considered significant. Representative results are shown (**, $>0.5\,cm^2$; *, $<0.5\,cm^2$).

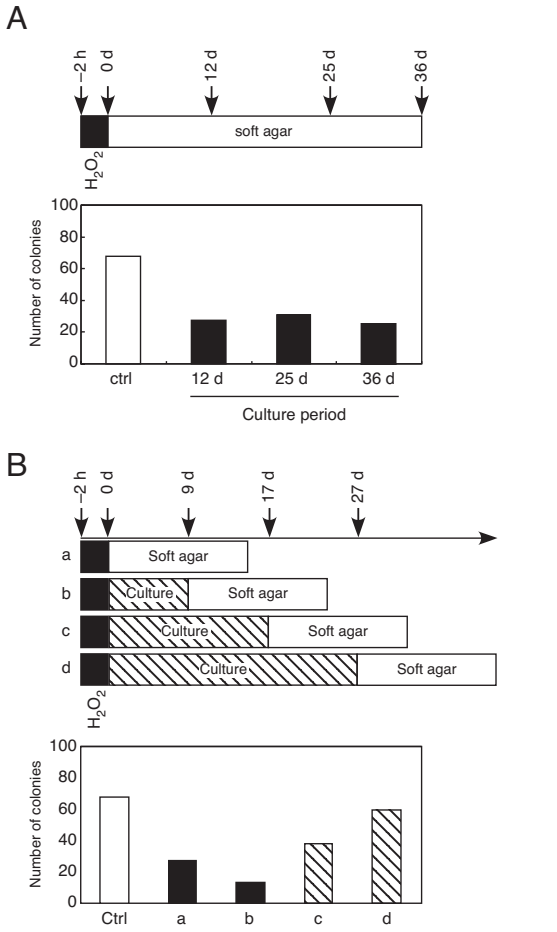

Fig. 3. Tumorigenicity of A549 Cells Treated with H_2O_2.
A, A549 cells were treated with 100 μM H_2O_2 for 2 h, seeded onto soft-agar plates, and cultured in the absence of H_2O_2 for 36 d. The number of visible colonies was counted at the times indicated. B, The effects of the length of recovery culture in the absence of H_2O_2 on the colony-formation ability of H_2O_2-treated A549 cells were investigated. After the recovery culture (a, day 0; b, day 9; c, day 17; and d, day 27), the cells were seeded onto soft-agar plates and cultured in the absence of H_2O_2 for 15 d. The number of visible colonies was counted. Independent experiments were done 3 times, and representative results are shown.

A549 cells were resistant or underwent reversal during recovery culture in normal medium; however, these cells did not propagate on soft-agar plates (Fig. 3A).

Cell population derived from A549 cells treated with H_2O_2

To further characterize the tumorigenicity phenotypes, we cloned two types of cells from H_2O_2-treated A549 cells using a cloning cylinder. One type consisted of enlarged, flattened cells, which were thought to be H_2O_2 sensitive (Fig. 4A, A549S). A549S cells were cloned 1 week after treatment. The other type included round cells, which propagated after long-term recovery culture of the H_2O_2-treated cells (Fig. 4A, A549R). A549R cells were cloned 3 weeks after treatment. We then tested for the tumorigenicity of these cells, as described above. As shown in Fig. 4B, the A549R cells showed high tumorigenicity, comparable to that of A549 cells, as evidenced by the colony-formation assay. Further, the A549R cells reduced in tumorigenicity, but formed tumors, as evidenced by the tumorigenicity assay using nude mice. In contrast, the A549S cells demonstrated reduced colony-formation ability and complete lack of tumorigenicity in the nude mice, indicating that the tumorigenicity of the A549R cells differed significantly from that of the A549S cells, and that the H_2O_2-sensitive phenotype of A549S cells was maintained in the long-term culture. Thus all these results suggest that the A549 cells included two types of cell populations: A549S cells and A549R cells. Since the number of colonies increased depending on the length of the recovery culture (Fig. 3B), A549R cells outnumber A549S cells in long-term recovery cultures in normal media.

Discussion

Sublethal oxidative stress is widely known to induce SIPS in normal human diploid fibroblasts.[1)] In this study, we investigated to determine whether sublethal oxidative stress would induce cellular senescence in human lung adenocarcinoma A549 cells as well as normal cells, and whether the tumorigenicity of the tumor cells would change after induction of SIPS. Cellular senescence limits the replicative capacity of cells, thus preventing the proliferation of cells that are at different stages of malignancy. The recent body of evidence suggests that induction of senescence can be exploited as a basis for cancer therapy.[14)]

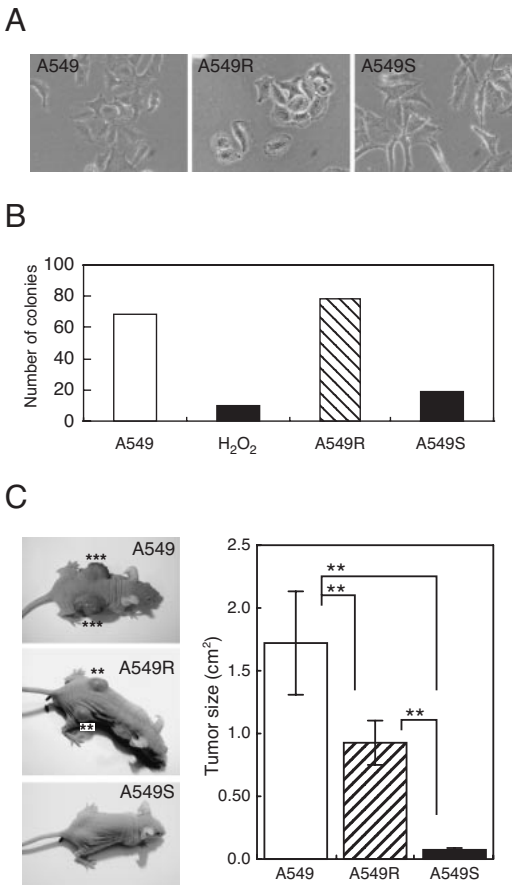

Fig. 4. Cell Population Derived from A549 Cells Treated with H_2O_2. A, Cell morphologies of A549, A549R, and A549S cells. B, Colony-formation abilities of A549R and A549S cells were tested as described above. Independent experiments were done 3 times, and representative results are shown. C, The tumorigenicity of A549R and A549S cells was tested using nude mice, as described above. Seventeen days after injection, the tumor size was measured. Tukey's all-pairwise comparison test was used to identify differences between A549, A549R, and A549S results. Differences at $P < 0.01$ were considered significant. Representative results are shown (tumor size: ***, $>1.0\,cm^2$; **, $>0.5\,cm^2$).

As shown in the results, H_2O_2 apparently triggered SIPS in most A549 cells, but in the long-term culture, H_2O_2-treated A549 cells were found to be of two types: one sensitive to H_2O_2 (A549S cells) and the other resistant or undergoing reversal (A549R cells). Although we could not precisely deduce the reasons, autonomous and microenvironmental factors might have been responsible for the existence of A549R cells among A549 cells in SIPS. Thus, factors such as the intrinsic ability of specific cells to buffer oxidative stress ultimately determine reversibility after oxidative stress. Further, very little is known about the molecular machinery that drives cells into an irreversible senescent state. Some authors distinguish two types of senescence-like arrests: a reversible one mediated by p53 and an irreversible one mediated by the concomitant actions of the INK4a/Rb and p53 pathways.[15] Thus the occurrence of A549R cells is partly explained by the fact that A549 cells and sublines contain almost the same levels of wild-type p53, pRb (Fig. 1D), and homozygous deletions of INK4a.[16,17] Further, some unidentified factors might compensate for the INK4a loss and irreversibly induce SIPS in A549S cells.

The inducibility of SIPS should be tested in other tumor cell lines, and the molecular basis of the irreversible induction of SIPS by H_2O_2 treatment of tumor cells should be further clarified. SIPS as well as oncogene-induced senescence in tumor cells are potential methods of tumor suppression, and an understanding of tumor senescence should make possible the development of new therapeutic approaches to improve the efficacy and decrease the side effects of cancer therapy.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References

1) Chen, Q., and Ames, B. N., Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblast F65 cells. *Proc. Natl. Acad. Sci. USA*, **91**, 4130–4134 (1994).
2) Gorbunova, V., Seluanov, A., and Pereira-Smith, O. M., Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. *J. Biol. Chem.*, **277**, 38540–38549 (2002).
3) Duan, J., Duan, J., Zhang, Z., and Tong, T., Irreversible cellular senescence induced by prolonged exposure to H_2O_2 involves DNA-damage-and-repair genes and telomere shortening. *Int. J. Biochem. Cell Biol.*, **37**, 1407–1420 (2005).
4) Zdanov, S., Debacq-Chainiaux, F., Remacle, J., and Toussaint, O., Identification of p38MAPK-dependent genes with changed transcript abundance in H_2O_2-induced premature senescence of IMR-90 hTERT human fibroblasts. *FEBS Lett.*, **580**, 6455–6463 (2006).
5) Toussaint, O., Medrano, E. E., and von Zglinicki, T., Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp. Gerontol.*, **35**, 927–945 (2000).
6) Toussaint, O., Remacle, J., Dierick, J. F., Pascal, T., Frippiat, C., Zdanov, S., Magalhaes, J. P., Royer, V., and Chainiaux, F., From the Hayflick mosaic to the mosaics of ageing: role of stress-induced premature senescence in human ageing. *Int. J. Biochem. Cell Biol.*, **34**, 1415–1429 (2002).
7) Frippiat, C., Chen, Q. M., Zdanov, S., Magalhaes, J. P., Remacle, J., and Toussaint, O., Subcytotoxic H_2O_2 stress triggers a release of transforming growth factor-beta 1, which induces biomarkers of cellular senescence of human diploid fibroblasts. *J. Biol. Chem.*, **276**, 2531–2537 (2001).
8) Katakura, Y., Nakata, E., Miura, T., and Shirahata, S., Transforming growth factor beta triggers two independent-senescence programs in cancer cells. *Biochem. Biophys. Res. Commun.*, **255**, 110–115 (1999).
9) Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C. A., Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*, **436**, 660–665 (2005).
10) Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W., Cordon-Cardo, C., and Pandolfi, P. P., Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*, **436**, 725–730 (2005).
11) Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M., Beach, D., and Serrano, M., Tumour biology: senescence in premalignant tumours. *Nature*, **436**, 642 (2005).
12) Michaloglou, C., Vredeveld, L. C., Soengas, M. S., Denoyelle, C., Kuilman, T., van der Horst, C. M., Majoor, D. M., Shay, J. W., Mooi, W. J., and Peeper, D. S., BRAFE600-associated

senescence-like cell cycle arrest of human naevi. *Nature*, **436**, 720–724 (2005).

13) Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi, J., A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl. Acad. Sci. USA*, **92**, 9363–9367 (1995).

14) Dimri, G. P., What has senescence got to do with cancer? *Cancer Cell*, **7**, 505–512 (2005).

15) Beausejour, C. M., Krtolica, A., Galimi, F., Narita, M., Lowe, S. W., Yaswen, P., and Campisi, J., Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.*, **22**, 4212–4222 (2003).

16) Iavarone, A., and Massague, J., Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15. *Nature*, **387**, 417–422 (1997).

17) Jia, L. Q., Osada, M., Ishioka, C., Gamo, M., Ikawa, S., Suzuki, T., Shimodaira, H., Niitani, T., Kudo, T., Akiyama, M., Kimura, N., Matsuo, M., Mizusawa, H., Tanaka, N., Koyama, H., Namba, M., Kanamaru, R., and Kuroki, T., Screening the p53 status of human cell lines using a yeast functional assay. *Mol. Carcinog.*, **19**, 243–253 (1997).