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Cellular Senescence Controlled by p53 is a Barrier to Environmental Carcinogenesis

Maria del Mar Vergel, Sandra Muñoz-Galvan, Daniel Otero-Albiol and Amancio Carnero*

Instituto de Biomedicina de Sevilla (IBIS), HUVR/Consejo Superior de Investigaciones Cientificas/Universidad de Sevilla, Sevilla, Spain

***Corresponding author**: Amancio Carnero , Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocio, Edificio IBIS Avda. Manuel Siurot s/n., 41013, Sevilla, Spain, E-mail: acarnero-ibis@us.es

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Abstract

Cancer development in humans and animals may be caused by environmental factors. It has been estimated that approximately 80% of human tumors are generated by exposure to environmental carcinogens. The carcinogens may initiate or induce progression of tumors in several ways. Cellular senescence is a natural barrier used by cells to respond to stress. The molecular analysis of immortal clones shows alterations, either structural or epigenetic, in the genes involved in cellular senescence. It is thought that these alterations are caused directly by mutating or methylating the genes involved in cellular senescence. Therefore, understanding cellular senescence and how it can be modified by environmental carcinogens may be essential to control the increase in cancer prevalence. In the present work, we explored the role of cellular senescence barrier in the carcinogenic potential of some known carcinogens. We found that most carcinogens tested induce a primary senescent response in diploid mouse embryonic fibroblasts (MEFs) and the clones arising with proliferative capacity contain mutated p53 protein. This primary response of senescence induction is abolished in the presence of the p53 inhibitor, pifithrin-a. Under these conditions, the tumorigenic potential of carcinogens is greatly increased. Upon elimination of pifithrin-a from the media, cellular senescence is restored. Therefore, the first cellular response to a carcinogen is a cell cycle arrest program that may result in a permanent arrest with features of cellular senescence. If there is a concomitant alteration of genes involved in cellular senescence, which promotes cellular immortalization, a further carcinogenic insult may increase the chances of tumorigenesis and a malignant clone may develop.

Keywords: Carcinogenesis; Cellular senescence; Tumorigenesis; Carcinogens

Introduction

Carcinogens are widespread in nature. Humans and animals have been exposed to carcinogens for millions of years, especially those in the external environment, such as food. It has been estimated that 80% of human tumors are generated by exposure to environmental carcinogens including chemicals, viruses, and non-ionizing and ionizing radiation [1,2].

Cancer development in humans and animals as a result of environmental factors, chemicals, viruses, radiation, and diet is a long process, requiring a large portion (from a third to half) of the lifespan of the organism [1,2]. It is now understood that several mutations need to accumulate in different pathways to result in a full tumorigenic phenotype [3,4]. Initiation with one of many different carcinogens is typically followed by the spontaneous or autonomous proliferation of cells intended to form a tumor. However, the autonomous or semiautonomous growth of initiated cells only occurs late in the carcinogenic process. Focal lesions with autonomous cell proliferation can only be observed after large doses of carcinogens and much longer periods of exposure than that required for initiation. In fact, virtually every chemical carcinogen is an inhibitor of cell proliferation [5,6]. Haddow has suggested that the inhibition of cell proliferation could be an early effect of carcinogens and that in such an environment resistant cells might arise and be encouraged to proliferate [7]. The growth of rare altered cells leading to focal neoplasms is a key phenomenon in the promotion of cancer development in virtually all experimental carcinogenesis models and in many human systems [1,3].

Different chemical agents, both mutagens and non-mutagens, have been shown to induce cellular senescence in primary cells [8-11]. Cellular senescence is a unique state of irreversible proliferative quiescence and terminal differentiation. It is characterized by changes in transcription, chromatin conformation, cytoplasmic and nuclear morphology, DNA damage signaling, and a strong increase in the secretion of pro-inflammatory cytokines [12-14]. Senescence is the first line of defense against potentially transformed cells that remain in a state of permanent proliferative arrest [15-17]. Progression to malignancy correlates with a bypass of cellular senescence [18]. Senescence has been observed in vitro and in vivo in response to various stimuli including oncogenic stress [19,20], oxidative stress [21], and chemotherapeutic agents [22,23]. Cells with cellular and molecular characteristics of senescence have been found to be associated with the activation of oncogenes and the inactivation of tumor suppressor genes in precancerous benign neoplasms in both humans and animal models [24-27].

Since the early 1980s, cellular senescence has been viewed as a barrier to tumorigenesis, and this has been demonstrated by the seminal work of Newbold and colleagues [28,29]. These and other authors have shown that it is necessary to bypass senescence to initiate immortal and/or tumoral clones from a naive culture.

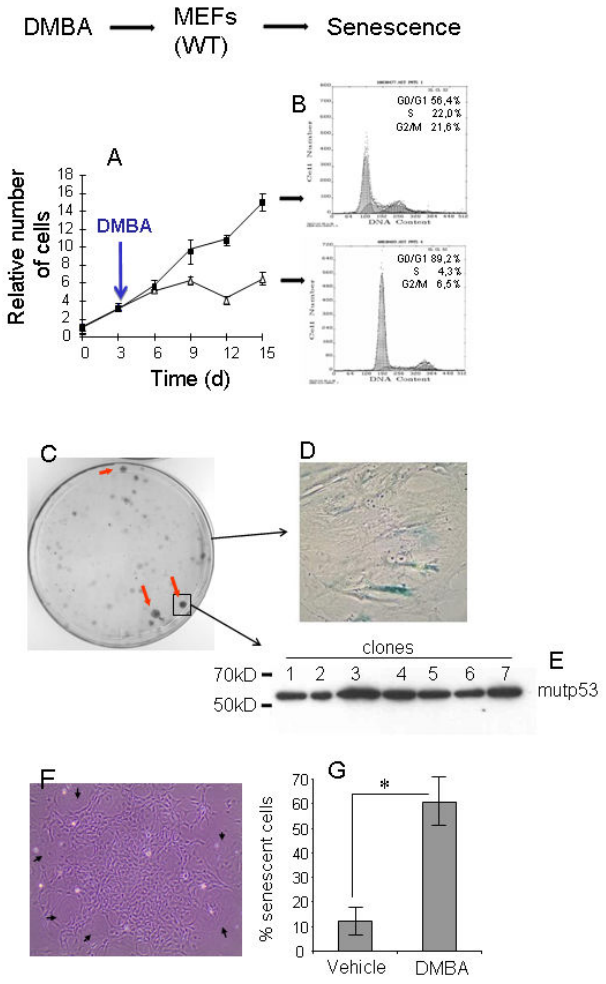


Figure 1: Effect of DMBA carcinogen in primary MEFs. A) Growth curve of primary cells (MEFs) treated DMBA alone (DMBA,), or untreated (). Arrow indicates the time of treatment with DMBA. B) FACS analysis of the proliferative status of the cells treated with DMBA (bottom figure), or vehicle only (upper figure). C) 104 MEFs were seeded in a 10 cm plate and treated with 10 M DMBA. After 7-10 days the cells were fixed and stained with crystal violet to identify clones. Arows indicate actively growing clones. D) Phenotype of cells treated with DMBA. E) Individual clones were subcultivated independently and the presence of p53 was analyzed by Western blot. Cells expressing mutant p53 (175H) were used as control. F) Picture of a representative actively growing clone from C. Arrows indicate senescent cells. G) Quantification of the percentage of senescent cells from 3 independent experiments. Senescent cells were processed as indicated in materials and methods and more than 400 cells counted in each experiment. Data shows the average of the senescent cells. Barrs show SD. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, Student's T test.

However, despite the highly increased ratio of immortalization induced by carcinogens, the vast majority of cells remained non-proliferative. The molecular analysis of immortal clones showed alterations, either structural or epigenetic, in the genes involved in cellular senescence [30,31]. It is thought that these alterations are caused directly by the carcinogens. This may lead to biased identification. Only carcinogens able to alter cellular proliferation and cause immortalization in parallel will produce tumors. Some examples of these types of carcinogens are non-specific mutagens or genome epigenetic modifiers.

In the present work, we analyzed the effect of carcinogen treatment on naive-presenescent cells and found that the carcinogens tested primarily induced cellular senescence. Furthermore, the chemical elimination of the senescent barrier greatly enhances their carcinogenic potential.

Results

To perform a proof of concept, we chose the chemical DMBA (7,12-dimethylbenz (a) anthracene), a carcinogen used as a tumor initiator due to its mutagenic potential. In parallel, we also chose a distinctly non-carcinogenic compound that specifically targets p53, pifithrin-a. Pifithrin-a is a small chemical molecule that specifically inhibits the transcriptional activity of p53 by binding to the active protein. Pifithrin-a is perceived as non-carcinogenic, and it has also been proposed that pifithrin-a can be used in combination with chemotherapy and/or radiotherapy to reduce side-effects from the toxicity of these treatments because this toxicity depends largely on p53 activity.

We chose murine embryonic fibroblasts (MEFs) from CD1 embryos as a model cell system for testing this initial concept. In culture, these MEFs can proliferate for 10 to 14 doublings, allowing us to study the induced early senescence.

First, we seeded 104 MEFs in triplicate in 2.5 cm diameter dishes. After 24 hours, the cells were treated with 10 microM DMBA or with vehicle alone (DMSO) (Figure 1A). After treatment, we observed that a rapid decrease in cell proliferation was initiated (Figure 1A). After 48-72 hours, the cells suffered an irreversible G1 arrest (Figure 1B and C) and acquired a senescence phenotype showing expression of SA-Gal activity (Figure 1D and 1G). After 1-3 weeks, 2-4 clones eventually emerged from each plate (Figure 1F), which is an immortalization efficiency of approximately $1^{-4}\times10^{-4}$. This immortalization efficiency is 10-fold higher than that of spontaneously immortalized untreated MEFs, which accounts for approximately 1-2×10-5. p53 protein analysis of the individual clones showed that all growing clones analyzed arising from the DMBA-treated plate had mutated p53 (Figure 1E). Therefore, DMBA carcinogenic treatment induced senescence in MEFs, and the cellular clones that escape the senescent barrier carry mutated p53. The same effect was observed in IMR90 primary human fibroblasts (data not shown).

The above experiments were repeated, but this time the cells were pre-treated with 10 microM pifithrin-a (Figure 2A). Treatment with this compound allowed the murine cells to have unlimited proliferative capacity (or at least more than 20 doublings while the experiment continued) (Figure 2B and 2C). These cells were unable to form tumors in nude mice or form clones in soft agar, confirming the non-carcinogenicity of this compound by itself. However, when DMBA was added to pifithrin-a pre-treated cells, these cells did not enter senescence (Figure 2D). In addition, the cells formed actively growing colonies (Figure 2E) with an efficiency of 10-15% (similar to the efficiency of immortal cell lines from mice or human tumors). The analysis of several of these colonies showed that none of them had

mutated p53 (Figure 2F), indicating that clones with mutations in p53 were not dominant in this setting. Furthermore, these clones formed colonies in soft agar (Figure 2G) although with different efficacy (Figure 2H). Therefore, the non-carcinogenic compound pifithrin-a significantly enhanced carcinogenesis, increasing the carcinogenic potential of DMBA by more than 1,000-fold.

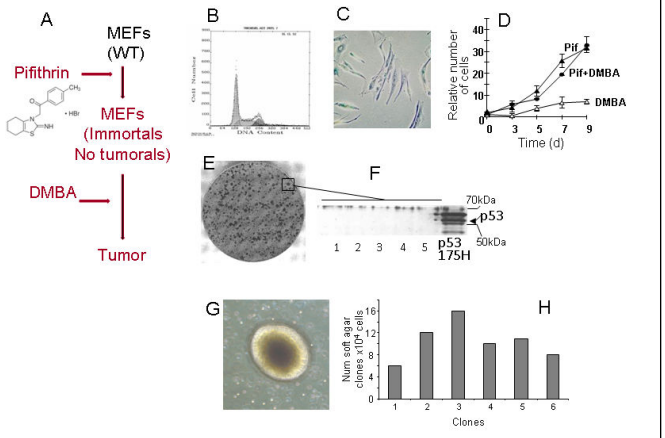


Figure 2: Proof of concept: non-carcinogenic compound pifithrin-a facilitates DMBA carcinogenesis. A) Schematic representation of the experiment. B) FACS analysis of the proliferative status of pifithrin-a-treated cells. C) Phenotype of pifithrin-a-treated cells. D) Growth curve of cells treated with pifithrin-only (Pif,) cells, DMBA alone (DMBA,) or pifithrin-a + DMBA (DMBA + Pif,). E) 104 MEFs were seeded in a 10 cm plate and were treated with 10 M pifithrin-a and 10 M DMBA. After 7-10 days the plate was fixed and stained with crystal violet to identify clones. F) Individual clones were subcultivated independently and the presence of p53 was analyzed by Western blot. Cells expressing mutant p53 (175H) were used as control. G) Photo of a colony of MEFs treated with pifithrin-a and DMBA growing in soft agar. *: p<0.05, **: p<0.01, ***: p<0.001, Student's T test. H) Number of soft agar growing colonies in 6 different clones.

Finally, to assess the causative role of senescence in the carcinogenic potential, we eliminated the p53 inhibitor pifithrin-a from the culture and analysed the outcome. The growing population of MEFs treated with DMBA arrested with signs of cellular senescence 48-72 hr after elimination of pifithrin-a from the media (Figure 3).

To explore whether this effect was unique to DMBA or was a common feature of other carcinogens, we performed similar experiments with Nickel chloride, Diethylstilbestrol, Reserpine, Phenobarbital, N-methyl-N-nitrosourea (MNU) and Benzo(a)pyrene. Early presenescent MEFs were treated with carcinogens in the presence or absence of pifithrin-a, and the phenotype was analyzed after 72 hr. Untreated MEFs showed robust proliferative capacity, while MEFs treated with carcinogens showed a decrease in proliferation that coincided with the appearance of senescence features (Figure 4). However, nickel chloride treatment showed a high percentage of apoptotic cells but no signs of senescence. In all cases, the carcinogen-induced effect was dependent on p53 because treatment with pifithrin-a decreased the senescence or apoptosis and increased the proliferative capacity of the cells (Figure 4).

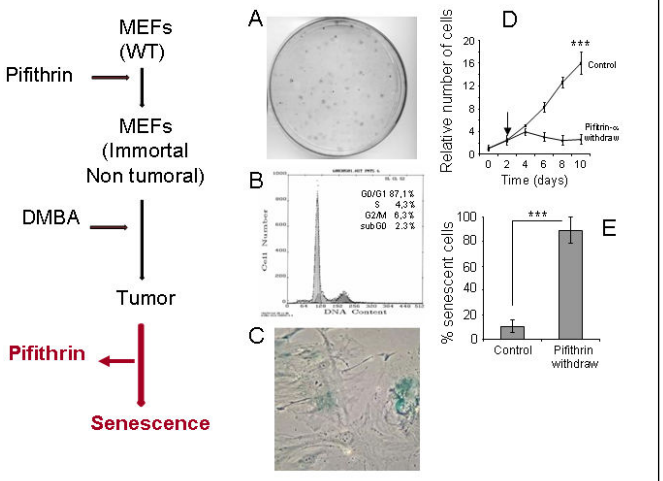
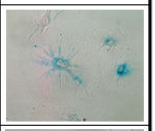
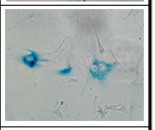
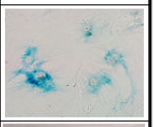


Figure 3: Elimination of pifithrin-a from the media restores senescence. Left) Schematic representation of the experiment. A) 104 MEFs were seeded in a 10 cm plate and were treated with 10 M pifithrin-a and 10 M DMBA. After 7 days pifithrin-a was eliminated from the media and cells grown for other 72 hrs in media without pifithrin-a. Then, the plate was fixed and A) stained with crystal violet to identify clones. B) processed to perform a FACS analysis of the proliferative status or C) analyze the phenotype of pifithrin-a- withdraw cells. D) Growth courve of cells trated with pifithrin-a (control), or in which pifitrhin-a was withdraw from the media. Arrow indicates the moment of the pifithin-a withdrawal. E) Cuantification of the percentage of senescent cells from 3 independent experiments. Senescent cells were processed as indicated in materials and methods and more than 150 cells counted in each experiment. Data shows the average of senescent cells. Barrs show SD *: p<0.05, **: p<0.01, ***: p<0.001, Student's T test.

Discussion

We have found that primary MEFs are induced to senesce by DMBA and other carcinogens, and that the transitory chemical bypass of senescence allows a great increase of the tumorigenic potential of the carcinogen. This is a common feature to many, but not all, the carcinogens tested. Therefore, the first cellular response to a carcinogen is a cell cycle arrest program that may result in a permanent arrest with features of cellular senescence. If there is a concomitant alteration of genes involved in cellular senescence, which promotes cellular immortalization, a further carcinogenic insult may increase the chances of tumorigenesis and development of a malignant clone. Thus, it seems that the first response to a "mutagenic stress" may be the induction of cellular senescence for most carcinogens, or apoptosis for some types of carcinogens such in nickel chloride. The cell becomes immortal only when this physiological barrier is inactivated and when a focal clone that can originate a tumor is initiated. We can speculate that cellular senescence is an evolutionary barrier developed to delay environmentally induced tumorigenesis.

Carcinogen	Senescence	(+ Pifithrin-α)	
Diethylstilbestrol (10 μM)	+ +	-	
Reserpine (5 μM)	+ +	-	
N-nitroso.N-methylurea (10 μM)	+ +	-	
Benzo[a]pirene (1 μM)	+ +	+/-	
Nickel chloride (0.5 μM)	- (High Apoptosis)	- (Low Apoptosis)	

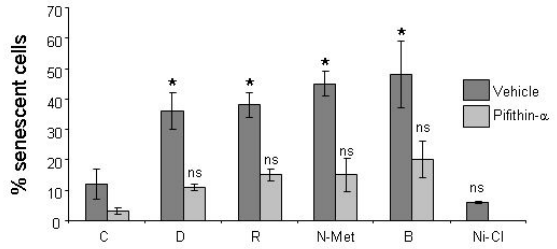


Figure 4: Figure showing the effect observed with several carcinogens tested in naïve MEFs. Upper table shows a resume of the results. Bottom graph shows the averaged quantification of three independent experiments. Barrs show SD *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, Student's T test. All values were compared to untreated control cells for statistical significance.

Therefore, we suggest that cellular senescence is the first response to environmental carcinogens. We also argue that inhibition of the cellular senescence process, even temporary, will trigger an increased immortalization effect leading to cancer initiation for many environmental carcinogens.

Recently, an *in vivo* system has been reported, based on the activation of p16INK4a [32], a classic marker activated upon cellular senescence [12,19,33]. The murine strain harbors a knock-in of the luciferase gene into the cdkn2a locus [34]. The model exposed to arsenic, cigarette smoke and UV light potently activated p16INK4a-mediated senescence [32].This in vivo data seems to corroborate our main conclusions. However, senescence is not the only physiological end point observed during environmental carcinogens stimuli. Ray and Swanson, reports that dioxin appears to accelerate differentiation of human epidermal keratinocytes [35]. Furthermore, dioxin appears to decrease SA-gal staining but increases the expression of p53, p16INK4a and p14ARF, cell cycle regulatory proteins [35] also involved in senescence [12,19,33]. Our own work shows that the response to nickel chloride is apoptosis and not senescence was observed. It is possible that the specific response varies among doses and tissues types. The fact that the response is dependent of p53 seems a general effect based in its role as DNA damage and stress sensor [36,37]. But in many cases cellular senescence has been reported to be induced in cells without p53 activity (see below).

The experiments reported here are proofs of concepts. Chemical adaptation studies with genetic alterations have been in the field since 1997 and confirmed the existence of chemical molecules that exist in the environment without being carcinogenic and perform an essential function allowing other molecules to become tumorigenic. For example, pifithrin-a was discovered in hospital environments in patients treated with chemotherapy and/or radiation therapy. It is also possible that other molecules with similar functions may exist in other environments with wider distribution in the population. Interestingly, the treatment of various tumor cell lines with different chemotherapeutic agents, radiation, or differentiating agents also induces irreversible growth arrest with features similar to cellular senescence [23]. Moderate doses of doxorubicin induced a senescent phenotype in 11 out of 14 tumor cell lines, independent of p53 status [38]. A similar effect has been observed in cell lines derived from human tumors treated with cisplatin [39], hydroxyurea [40], and bromodeoxyuridine [41], which are all DNA-damaging agents. The propensity of tumor cells to undergo senescence in response to damage induced by commonly used chemicals was compared in cell lines from various origins [23]. Under equitoxic doses, the strongest induction of a senescent phenotype was observed with DNA-interacting agents (doxorubicin, aphidicolin, and cisplatin). The weakest effect was observed with microtubule-targeting drugs (taxol and vincristine). A moderate response was observed with ionizing radiation, cytarabine, and etoposide. The induction of senescence by the drugs was dose dependent and correlated with the growth arrest observed in culture [8,40-42]. Drug-induced senescent phenotypes have been confirmed in vivo ([22,43,44] and references therein).

Most chemical used in cytotoxic chemotherapy against cancer are mutagens. It is necessary to consider the possible by-side effect by combination with modifiers of senescence. This aspect might have a great clinical relevance and also for the regulation of carcinogenic doses and non-carcinogenic substances with potential to contribute to cancer.

Senescence is a mechanism imposed to limit the number of divisions that somatic cells can undergo before being permanently arrested. This mechanism has a high degree of redundancy. Moreover, system insults to prevent senescence are generally recognized as an unwanted signal, which also triggers a senescence response. Our current knowledge is an interpretation of experimental designs in which acute molecular or cellular changes occur. There are very few experiments in which the effects of chemical compounds are analysed in combination with a senescence suppressor, or at low doses that cause chronic stress. There are even fewer experiments that take into account the different cellular and molecular contexts that may arise during the lifetime. Design models and cellular systems of the body that allow these types of tests are necessary to further explore the effects of environmental chemical carcinogens.

In summary, our work shows that the primary response to most environmental carcinogens in naïve cells is cellular senescence, and non-carcinogens able to alter the senescent response greatly increases the carcinogenic potential of these substances.

Materials and Methods

Cell cultures

Murine embryonic fibroblasts (MEFs) were kindly provided by the Laboratories of Marcos Malumbres and Carmen Blanco-Aparicio at CNIO. The cells were cultured in DMEM supplemented with 10% serum, antibiotics and antifungicides.

Growth curve

MEFs were seed at a density of 104 cells in 2.5 cm dishes in triplicate samples. After 18 h, medium was changed (day 0) and fresh media added. Every 2 days cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 15% acetic acid and quantified at 595 nm as a relative measure of cell number. Values are expressed as the relative number of cells growing related to day 0.

Clonability assay

A total of 10,000 cells was seeded in triplicate 10 cm plates and allowed to grow under the conditions indicated above for 7-10 days. After this time, the plates were fixed with 0.5% glutaraldehyde and stained with 0.2% crystal violet.

SA-b-galactosidase

Senescence-associated (SA) β-galactosidase (β-Gal) activity was measured as previously described [45], except that cells were incubated in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X Gal) at pH 5.5 to increase the sensitivity of the assay in MEFs. The percentage of cells expressing SA ß-Gal was quantified by inspecting >400 cells per 10 cm diameter plate three times.

Determination of p53 by western blot

Cells were prepared in lysis-buffer and proteins were separated on SDS-PAGE gels, transferred onto PVDF membranes (Immobilon-P, Millipore) and immunostained. The primary antibody used was anti-p53FL393 (Santa Cruz 6243, diluted 1:1000), and the secondary antibody was horseradish peroxidase-labeled goat anti-rabbit (Calbiochem 401315, diluted 1:4000). Proteins were visualized using the ECL detection system (Amersham Biosciences).

Annexin staining

Exponentially growing cells (106) were incubated in DMEM + 10% FBS, and 24 h later, the detached cells in the supernatant were obtained and mixed with trypsinized cells as indicated [46]. They were centrifuged for 5 min at 1100 rpm and the pellets were washed with PBS and resuspended in 1X binding buffer (BD Pharmingen; 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Then, cells were incubated with 5 µl annexin V (BD Pharmingen) and 10 µl propidium iodide (Sigma) for 15 min in the dark. Flow cytometry (FACs) analysis was performed using a Becton and Dickinson FACScalibur cytometer and data were analyzed with Cell Quest Pro software.

Soft Agar Assay

To measure the anchorage-independent growth [47], 2 × 104 cells were suspended in 1.4% agarose D-1 Low EEO (Pronadisa) growth medium containing 10% FBS, disposed onto a solidified base of growth medium containing 2.8% agar (agarose D-1 Low EEO, Pronadisa) and overlaid with 1 ml of growth medium. After 24 h, media containing 10% FBS was added to each 35 mm dish and renewed twice weekly. Colonies were scored 3 weeks later, and all values were determined in triplicate. Photographs were taken with a phase-contrast microscope (Olympus).

Acknowledgments

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