Sensitivity of Neoplastic Cells to Senescence Unveiled Under Standard Cell Culture Conditions

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Abstract. *Background: Cancer cells are typically defined as infinitely proliferating, whereas normal cells (except stem cells) are considered as being programmed to become senescent. Our data show that this characterization is misleading. Materials and Methods: Multiplex Ligation-dependent Probe Amplification, TP53 sequencing, real-time polymerase chain reaction (PCR) for MUC1 and SCGB2A2 and immunocytochemistry, together with senescence detection assay and real-time microscopic observations were used to analyze primary neoplastic cells isolated from prostate, breast and colorectal tumors, as well as stable cancer cell lines (MCF7, MDA-MB-468, SW962, SK-MEL28, NCI-H1975 and NCI-H469). Results: In all cases of primary cancer cell cultures, in vitro conditions rapidly revealed senescence in the majority of cells. Two out of six stable cancer cell lines did not exhibit any senescence-associated-β-Galactosidase-positive cells. Interestingly, four cell lines had small sub-populations of senescent cells (single SA-β-Gal-positive cells). Conclusion: Primary neoplastic cells from different types of cancer (prostate, breast, colon cancer) appear to be senescent in vitro. Apparently, cancer cell lines that have been used for many years in drug-testing analyses have constantly been misleading researchers in terms of the general sensitivity of cancer cells to senescence.*

Proliferation is a consequence of activation of growth pathways (BRAF, AKT, *etc.*) and inhibition or lack of cell-cycle blockers (p16, TP53, *etc.*), quiescence is an outcome of cell-cycle inhibition or lack of growth signals, while senescence results from activation of growth pathways and blockade of the cell cycle (1).

Textbooks describe cancer cells as being able to divide infinitely, however, such a view is mostly based on analyses of stable cancer cell lines. Surprisingly, the majority of cancer cells cannot actually be cultured continuously *in vitro*. Nevertheless, not only has this fact tended to be ignored but the process of senescence is also not considered as a conceivable reason for failure of cell line stabilization, due to the widely accepted opinion that characteristic features of cancer cells include a limitless proliferative potential and resistance to senescence. However, since it was proven that hyperactivation of several oncogenes may lead to cell senescence when the cell cycle is stopped by a physiological mechanism (2), such an opinion was slightly undermined. A typical explanation for lack of senescence in neoplastic cells could be that simultaneous activation of oncogenes and inactivation of cell-cycle tumor suppressors reduces the pro-senescence arm of oncogene transduction pathways, and enables only pro-proliferative actions of mutated KRAS, BRAF, AKT and many other proteins encoded by oncogenes (3). Senescence has been shown to be a very important process at the early stages of tumorigenesis. When observed in cancer cells, it is mostly described as a mechanism depending on cytotoxic compounds, severe DNA damage or reactivation of suppressors (4, 5). Despite the widely accepted view that cancer cells can become senescent only in an extremely harmful environment, we detected that even typical *in vitro* conditions can trigger certain mechanisms which inhibit the cell cycle and unveil pro-senescence sensitivity of glioblastoma cells (6, 7). Moreover, we have lately described some intriguing discrepancies between cancer cell lines and tumor specimens (8). In the present study, we analyzed cancer cells freshly obtained from malignant tumor specimens (breast, prostate, colon) and cancer cell lines for their senescence sensitivity *in vitro*.

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Materials and Methods

Tissue samples and primary cell culture. Primary cell cultures, obtained from five patients diagnosed with breast cancer and five patients diagnosed with colorectal cancer, and DNA isolated from corresponding frozen samples were purchased from Celther Polska, Ltd, (Lodz, Poland). Prostate tissue samples were obtained from 10 patients diagnosed with prostate cancer (treated at the Clinic of Urology, Medical University of Lodz). All samples were collected using the protocol approved by the Bioethical Committee of the Medical University of Lodz (Approval No. RNN/118/11/KE). Written informed consent was obtained from all patients and their data were processed and stored according to the principles expressed in the Declaration of Helsinki. The patients were diagnosed according to the World Health Organization Criteria. Tumor specimens were shipped in 1× Hank's Balanced Salt Solution (HBSS; PAA, The Cell Culture Company, Pasching, Austria). Irrespective of cell culture type, isolation of cells from fresh tumor specimens started within 3 hours after surgery.

Establishment and growth of prostate cancer cells under classical culture conditions. Freshly resected human tumor tissue samples were washed in HBSS and minced into small pieces with scalpels, followed by enzymatic dissociation. Prostate tissue pieces were dissociated using collagenase type IV (200 U/ml; Sigma-Aldrich, St. Louis, Missouri, USA) and dispase (5 mg/ml) at 37˚C overnight. To achieve a single prostate cell suspension, pellet was pipetted with trypsin-EDTA (0.25%) and then with solution of dispase (5 mg/ml) and DNase I (1 mg/ml). Primary prostate cancer cells thus obtained were cultured on bovine collagen-coated 6-well plates in PrEGM™ (Lonza, Basel, Switzerland).

Breast and colon primary cell culturing. Primary breast and colon cancer cell cultures were established in Celther Polska Laboratory according to the following protocol: freshly resected human tumor tissue samples were washed in HBSS and minced into small pieces with scalpels, followed by enzymatic dissociation. Colonic tissue fragments were dissociated using type I collagenase (600 U/ml; Gibco, BRL, Lyon, France) and dispase (5 mg/ml), cell suspensions were collected twice: first time after 2 h incubation at 37˚C and a second after subsequent overnight incubation at 4˚C. Minced breast tumor tissues were processed according to the procedure described by Stem Cell Technologies, (Vancouver, Canada). Briefly, tissue samples were dissociated using 1× collagenase/hyaluronidase and incubated overnight at 37˚C. After centrifugation, to achieve a single mammary cell suspension, the cell pellet was suspended with trypsin-EDTA (0.25%) and then with a solution of dispase (5 mg/ml) and DNase I (1 mg/ml).

Primary breast and colonic cancer cells were cultured on bovine collagen-coated 6-well plates in appropriate culture medium: colonic cells in AR-5 medium [according to the protocol by Park *et al*. (9)] and mammary cells in EpiCult™ - C Human Medium Kit (Stem Cell Technologies).

Cell lines. Commercially available human cancer cell lines MCF7, MDA-MB-468, SW962, SK-MEL28, NCI-H1975 and NCI-H460 were obtained from the American Type Culture Collection (ATCC) Manassas, VA, USA. NCI-H1975 and SW962 cells were cultured in RPMI-1640 medium (PAA, Linz, Austria), MCF7, SK-MEL28 and NCI-H1975 cells were cultured in MEM (PAA) and MDA-MB-468 cells were cultured in DMEM/F12 (PAA). Each medium was supplemented with 10% FBS (PAA) and penicillin/ streptomycin/gentamicin (GIBCO BRL, Paisley, UK). Cells were cultured in 5% CO_2 and passaged with trypsin-EDTA (0.05% trypsin; Gibco).

DNA/RNA isolation. DNA and RNA were isolated from frozen surgical samples (stored at −80˚C), corresponding primary cell cultures and colonies of cells exhibiting epithelial morphology. Additionally, nucleic acids were isolated from colonies showing features of senescence, collected by means of light microscopy with the use of a 100 µl pipette. Total cellular DNA and RNA were isolated using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA and DNA concentrations were measured spectrophotometrically and 100 ng of total RNA was reverse-transcribed into single-stranded cDNA using a QuantiTect Rev. Transcription Kit (Qiagen) according to the manufacturer's protocol.

TP53 sequencing analysis. *TP53* gene mutations were analyzed in exons 5 to 8. The primers used for the PCR amplification of cDNA sequences were: GTGCAGCTGTGGGTTGATT (exons 5-8, forward) and GCAGTGCTCGCTTAGTGCTC (exons 5-8, reverse); the annealing temperature was 53˚C. The sequencing primers were GCCATCTACAAGCAGTCACA (exons 5-8, forward) and CCC TTTCTTGCGGAGATTCT (exons 5-8, reverse). cDNA sequencing was performed using BigDye Seq kit v3.1 (Applied Biosystems, Foster City, CA, USA) and sequences were analyzed with an ABI 3130 genetic analyzer and DNA Sequencing Analysis Software (Applied Biosystems).

Multiplex Ligation-dependent Probe Amplification (MLPA). The MLPA reactions were performed using the commercially available probe mixes: P175 (Tumor-Gain), P294 (Tumor-Loss), P105 (Glioma-2), P173 (Gain-3) and kits (MRC, Rotterdam, Netherlands) according to the manufacturer's protocol. In brief, 5 µl samples containing 50–250 ng of genomic DNA were denatured at 98˚C for 5 min and then cooled to 25˚C. Next, 3 µl of hybridization mastermix (containing 1.5 µl of MLPA buffer and 1.5 µl of probe mix per sample) was added to each sample and incubated at 98˚C for 1 min and at 60˚C for 16 to 20 h. Next, without removing the tubes from the thermocycler (paused at 54˚C), 32 µl of ligase mastermix (containing 25 µl water, 3 µl ligase buffer A, 3 µl ligase buffer B and 1 µl ligase per sample) was added to each sample and incubated at 54˚C for 15 min and at 98˚C for 5 min, then cooled to 20˚C. Finally, 10 µl of polymerase mastermix (containing 7.5 µl water, 2 µl SALSA PCR primer mix and 0.5 µl polymerase per sample) was added to each sample and the cycling conditions were as follows: 35 cycles of denaturation at 95˚C for 30 sec, annealing at 60˚C for 30 sec and elongation at 72˚C for 60 sec followed by incubation at 72˚C for 20 min. The products were cooled to 15˚C and stored in a dark box at 4˚C. The fragments were separated by capillary electrophoresis using an ABI 3130 genetic analyzer (Applied Biosystems). The comparative analyzes were performed using Coffalyzer.Net v131211.1524 (MRC-Holland) and the resultant ratio for a given gene of more than 1.3 was interpreted as a gain, while that of less than 0.7 as a loss.

Real-time PCR for MUC1 and SCGB2A2. Real-time qPCR was performed using StepOnePlus Real-Time PCR System instrument (Applied Biosystems). PCR products were synthesized from cDNA

Table I. *Primer sequences used for real-time polymerase chain reaction analysis.*

Gene	Primer	Sequence
Hypoxanthine phosphoribosyltransferase 1	HPRT1_F	5'-TGAGGATTTGGAAAGGGTGT-3'
	HPRT1_R	5'-GAGCACACAGAGGGCTACAA-3'
TATA box binding protein	TBP_F	5'-GAGCTGTGATGTGAAGTTTCC-3'
	TBP_R	5'-TCTGGGTTTGATCATTCTGTAG-3'
Secretoglobin, family 2A, member 2	SCGB2A2_F	5'-TGCTGATGGTCCTCATGCTG-3'
	SCGB2A2_R	5'-ACACTTGTGGATTGATTGTCTTGG-3'
Mucin 1	MUC1_F	5'-GCTGCTCCTCACAGTGCTTA-3'
	MUC1_R	5'-TGGGTAGCCGAAGTCTCCTT-3'

Table II. *Primary and secondary antibodies (AB) used for immunocytochemical staining.*

AB	Host	Manufacturer	Dilution
Primary			
Anti-EGFR	Mouse	Santa Cruz Biotechnology, Inc., sc-120	1:100
Anti-TP53	Rabbit	Santa Cruz Biotechnology, Inc., sc-6243	1:100
Anti-mammaglobin A	Mouse	Santa Cruz Biotechnology, Inc., sc-398405	1:50
Secondary			
Anti-mouse Alexa Fluor®594	Donkey	Molecular Probes, Invitrogen	1:500
Anti-rabbit Alexa Fluor®488	Donkey	Molecular Probes, Invitrogen	1:500

EGFR, Epidermal growth factor receptor; TP53, tumor protein p53.

samples using the SYBR® Select Master Mix. Each sample was amplified in triplicate in a total reaction volume of 12 μl containing SYBR® Select Master Mix (2×), 200 nM of both forward and reverse primers primer, and 50 ng of cDNA. *HPRT1* and *TBP* genes were used as reference genes to normalize the expression level of the target gene. *SCGB2A2*- and *MUC1*-specific primers were used for amplification of the tested genes (Table I) The cycling conditions were as follows: 2 min at 50˚C (UDG activation), 10 minutes at 95˚C (polymerase activation) followed by 40 cycles of: 15 seconds at 95˚C (denaturation), 30 sec at 60˚C (annealing) and 30 sec at 72˚C (extension). To confirm the specificity of the amplification signal, the gene dissociation curve was considered in each case. Normalized relative expressions of *SCGB2A2* and *MUC1* genes in the tested samples *versus* control sample were calculated utilizing the method described by Pfaffl *et al*. (10), based on each sample's average Ct value and each gene's average PCR efficiency. No template control reactions were used to identify PCR contamination. cDNA from MDA-MB-468 breast cancer cells (ATCC) was used as the positive control to normalize the calculation of expression levels of *SCGB2A2* and *MUC1* genes. cDNA derived from dermal fibroblasts (ATCC) was used as a negative control.

Immunocytochemistry. For the immunocytochemical analyses, spheroid and monolayer cell cultures were fixed in 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 for 10 minutes at room temperature. Non-specific binding sites were blocked by incubation with 2% donkey serum (Sigma) in PBS for 1 h. For double or triple immunolabeling, fixed cells were subsequently incubated with the appropriate primary antibodies (Table II) for 1 hour at room temperature. Double or triple labeling was visualized by simultaneous incubation with a combination of species-specific fluorochrome-conjugated secondary antibodies (1 h, room temperature) (Table II). The control samples were incubated with the secondary antibodies alone and were otherwise processed identically. The slides were mounted with ProLong® Gold Antifade Reagent or ProLong® Gold Antifade Reagent with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA, USA) coverslipped and examined using Nikon Eclipse Ci-S (Melville, NY, USA) fluorescence microscope.

Methods used to analyze senescence. For the purpose of the study, we applied a method based on the combination of two techniques, SA-β-Gal staining and immunofluorescence, to determine the phenotype of cells with features of senescence at the single cell level (6). SA-β-Gal activity was recognized as the hallmark of senescence.

Senescence associated (SA)-β-Galactosidase staining: SA-β-Gal staining was performed basing on the protocol by Dimri *et al*. (11). Cells were washed three times with PBS and fixed with cold 3% paraformaldehyde for 5 minutes. Cells were then washed twice with PBS for 5 min. A fresh senescence-associated staining solution [1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, X-Gal in dimethylformamide (stock 20 mg/ml)/40 mM citric acid/sodium phosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$], pre-warmed to 37˚C, was then added, followed by incubation at 37˚C (no CO_2) for 12 hours. After the incubation, cells were washed twice with PBS for 5 minutes and photographed using an Olympus CKX41 microscope (Center Valley, PA, USA). The percentage of positively stained cells was subsequently calculated.

Table III. *Genetic alterations of analyzed primary cancer cell cultures and cancer cell lines.*

No	Case no	TP53	RB	CDKN2A	Other
1	PC1	c.239;AAC>GAC; Asn>Asp	ND	ND	NA
2	PC2	ND	ND	ND	del.: *NTRK1, ERBB4, CYP27B*; amp.: *PIK3CA*
3	PC3	ND	ND	ND	amp.: *MDM2, MYO5B*
4	PC4	ND	ND	ND	ND
5	PC5	ND	ND	ND	del.: IGF3
6	PC6	ND	ND	ND	ND
7	PC7	ND	ND	ND	del.: *BRAF, IGF3*; amp.: *NTRK*
8	PC8	ND	ND	ND	del.: *IGF1R3*; amp.: *NTRK, BRAF*
9	PC9	ND	ND	ND	del.: *IGF3, BRAF*
10	PC10	ND	ND	ND	del.: *IGF1R3*; amp.: *NTRK1*
11	BC1	ND	ND	ND	ND
12	BC2	ND	ND	ND	ND
13	BC3	ND	ND	ND	del.: *BRAF, NTRK, ERBB4*; amp.: *IRS2, IGF1R*
14	BC4	ND	ND	ND	del.: *MDM2*
15	BC5	ND	ND	ND	del.: *BRAF, NRAS, IGFB, IRS*; amp.: *NTRK1, NFKB, FGF4, IGF1R*
16	CC1	c. 134; TTT>CTT, Phe>Leu	ND	ND	del.: *BRAF, JAK*; amp.: *NTRK1, FGF4, IGF1R*
17	CC2	c.175; CGC>CAC, Arg/His	ND	ND	del.: *BRAF, NRAS1, JAK*; amp.: *FGF4, IGF1R*
18	CC3	c.175; CGC>CAC, Arg/His	ND	ND	NA
19	CC4	ND	ND	ND	del.: *SERPINB*; amp.: *IRS2, NTRK, PDGFRA, PIK3CA*
20	CC5	ND	ND	ND	ND
21	NCI-H1975 (lung adenocarcinoma)	Homozygous (818 G>A)	ND	Homozygous (205 G>T)	*ATR* (DNP); *EGFR* (2369 C>T; 2573 T>G); PIK3CA (353 G>A)
22	MDA-MB 468 (breast adenocarcinoma)	Homozygous (818 G>A)	Deletion	ND	*PTEN* (253 G>T)
23	SK-MEL 28 (malignant melanoma)	Homozygous (434-435 TG>GT)	ND	ND	*PTEN* (A>G); BRAF (1799 T>A); *EGFR* (2257 C>T)
24	SW 962 (carcinoma of vulva)	Heterozygous (797 G>T)	ND	ND	ND
25	MCF 7 (breast cancer)	ND	ND	ND	*PIK3CA* (1633 G>); *MAP3K13* (1138 G>A)
26	NCI-H460 (large-cell carcinoma)	ND	ND	ND	*PIK3CA* (1633 G>A); *MAP2K1* (401 A>G); *KRAS* (183 A>T)

Sources: MLPA and TP53 sequencing analyses for primary cell cultures (samples 1-20); Cancer Cell Line Encyclopedia (CCLE) and ATCC Cell Lines by Gene Mutation database for cancer cell lines (cases 21-26) [12,13]. PC: Prostate cancer; BC: breast cancer; CC: colorectal cancer; del: deletion; amp: amplification; ND: not detected. NA: not analyzed. NTRK1, neurotrophic tyrosine kinase, receptor, type 1; ERBB4, erb-b2 receptor tyrosine kinase 4; CYP27B, cytochrome P450, family 27, subfamily B, polypeptide 1; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; MDM2, MDM2 proto-oncogene, E3 ubiquitin protein ligase; MYO5B-myosin VB, IGF3-insulin-like growth factor 3, IGF1R-insulin-like growth factor 1 receptor, BRAF-B-Raf proto-oncogene, serine/threonine kinas, IRS2-insulin receptor substrate 2; NRAS-neuroblastoma RAS viral (v-ras) oncogene homolog; NFKB, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; FGF4, fibroblast growth factor 4; JAK, Janus kinase; SERPINB, serine (or cysteine) peptidase inhibitor, clade B; PDGFRA, platelet-derived growth factor receptor, alpha polypeptide; ATR, ATR serine/threonine kinase; PTEN, phosphatase and tensin homolog; MAP3K13, mitogen-activated protein kinase kinase kinase 13; KRAS, Kirsten rat sarcoma viral oncogene homolog.

Senescence-associated heterochromatin foci (SAHF): As a second marker of senescence, the presence of SAHF was analyzed. SAHF detection was possible by visualization of cell nuclei stained with DAPI and examined using a Nikon Eclipse Ci-S fluorescence microscope.

Real-time phase microscopy monitoring of primary cell culture. For the assessment of the proliferation rate of primary cancer cells, certain cases were subjected to *in vitro* real-time microscopic observation which was performed using a JuLi Smart Fluorescent Live Cell Imager (Bulldog Bio Inc. Portsmouth, NH, USA). Images were taken inside an incubator every 12 hours for 5-7 days depending on the case.

Statistical analysis. The statistical analyses were performed using STATISTICA 10.1 software (StatSoft, Tulsa, OK, USA). For the analysis of cell biological characteristics, the Mann–Whitney *U*-test (with α equal to 0.05) was applied to assess the differences between several types of neoplastic cancer cell cultures. Statistical analysis was used to compare the percentage of SA-β-Gal-positive cells among three primary cell cultures (prostate, breast and colon cancer) and six stable cell lines (MCF7, MDA-MB-468, SW962, SK-MEL28, NCI-H1975 and NCI-H469). The average percentage was obtained by analyzing 200 cells per case from passages 3-4 of at least four cases from each type of cancer in primary cell culture.

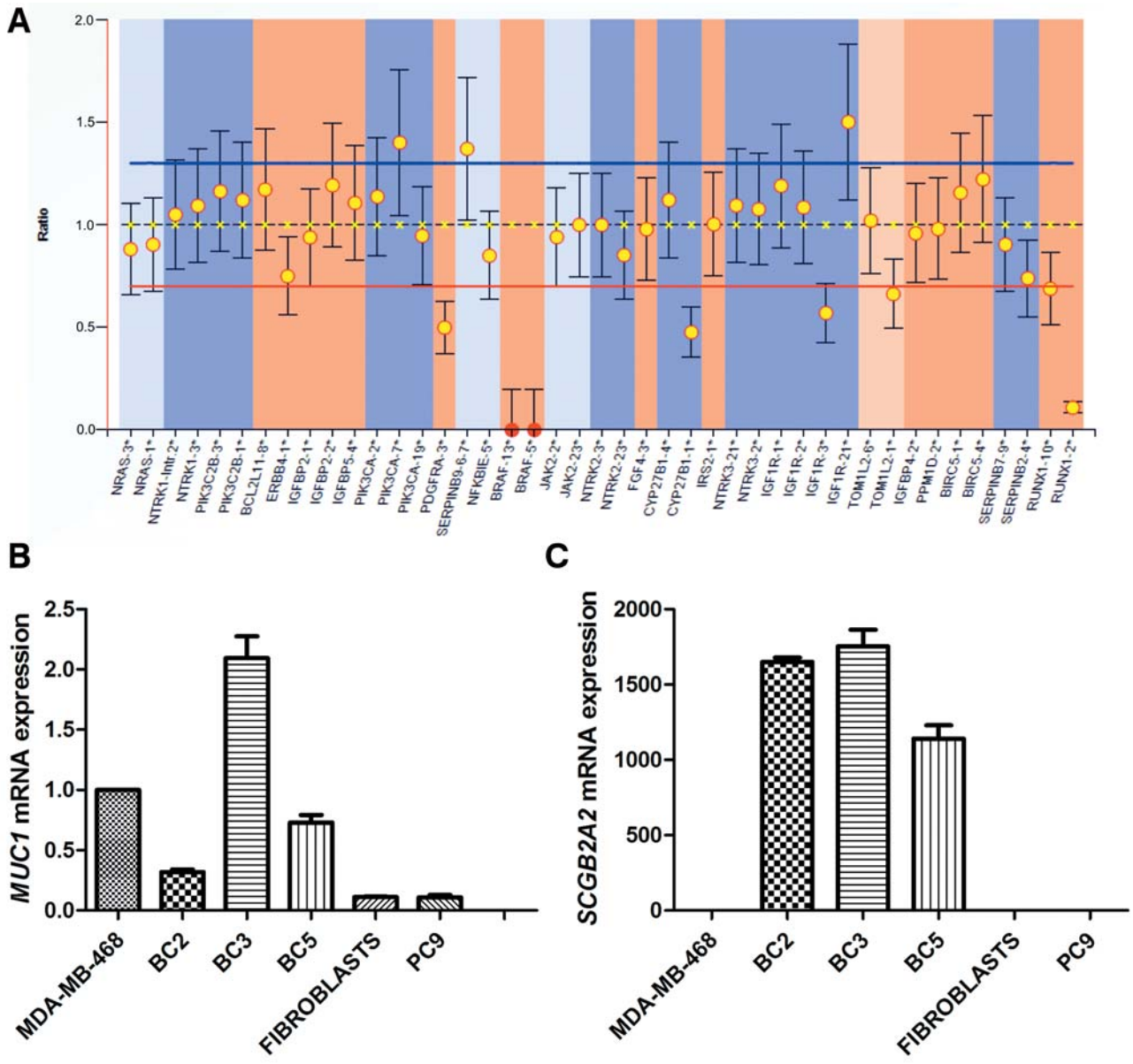

Figure 1. *Example of molecular analyses of primary cell cultures. A: Multiplex Ligation-dependent Probe Amplification (P173 kit) analysis of colorectal cancer cells (case no. CC2) revealed homozygous BRAF deletion. B, C: Real-time polymerase chain reaction (RT-PCR) analysis for MUC1 and SCGB2A2 expression in primary breast cancer cell cultures. Molecular characterization of every cancer cell culture analyzed confirmed their neoplastic origin.*

Results

Molecular characteristic of cancer cell lines and primary cell cultures. All analyzed samples were molecularly characterized (Table III and Figure 1). It is important to realize that molecular confirmation of senescence in cancer (not normal) cells was possible mainly on the basis of genetic changes detected in samples. If the first molecular change was detected (confirmation of tumor origin), further molecular analyses were abandoned. Thus, the percentage of changes does not follow the typical proportions of genetic alterations in analyzed tumors. In four out of 20 cases, we did not detect any molecular alterations in frozen samples by means of MLPA and *TP53* sequencing (Table III). For such genetically silent cases, we were unable to confirm beyond any doubt that senescent cells were neoplastic. To confirm the presence of breast cancer cells in primary culture, the expression of *MUC1* and *SCGB2A2* was analyzed (Figure 1B and C).

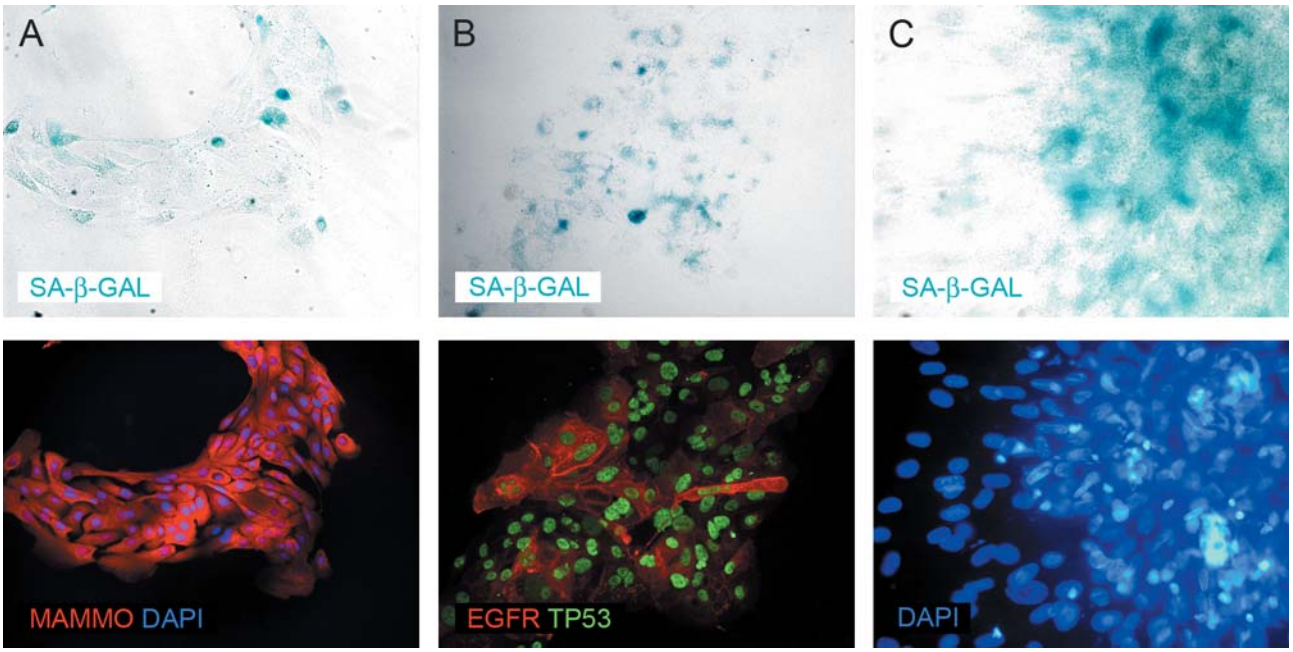

Figure 2. *Senescence analysis of primary cell cultures. A: Senescent breast cancer cell colony (case no. BC3; passage 3) expressing mammaglobin A. B: Colony of prostate cancer cells (case no. PC2 in fourth passage) showing increased SA-β-Gal activity in TP53 and EGFR-positive cells. C: Colorectal cancer cells (case no. CC4; passage 2) SA-β-Gal is present. Senescence-associated heterochromatic foci (SAHF) were not detected in SA-β-Gal-positive primary cancer cells. The images at the top and bottom present the same microscopic field.*

In vitro spontaneous senescence in primary cancer cells and cell lines. Senescence was detected using two markers: SA-β-Gal activity (by SA-β-Gal assay) and the occurrence of SAHF in cell nuclei (by DAPI microscopic visualization). Moreover, it was confirmed by real-time microscopic analyses.

All analyzed types of primary cancer cell (prostate cancer, breast cancer and colorectal cancer) had a high percentage of SA-β-Gal-active cells (Figure 2). Owing to the combination of two techniques (SA-β-Gal staining and immunofluorescence), we were able to determine the phenotype of cells exhibiting features of senescence at the single-cell level. Blue-stained cells (SA-β-Gal-positive) showed expression of specific cancer cell proteins, *e.g.* in breast cancer cell culture, mammaglobin A was detected (red signal; Figure 2A). In turn, in prostate cancer cell culture, neoplastic cells were identified by nuclear accumulation of TP53 (green signal) together with high expression of EGFR (red signal) (Figure 2B). The neoplastic origin of colonic cancer cells presented in Figure 2C was confirmed through MLPA analysis which indicated *BRAF* deletion.

Real-time microscopic observations performed for several cases (case no. BC2, PC3, PC4, CC1) confirmed that the majority of primary cancer cells stop dividing in early passages. Moreover, these observations revealed changes in cell morphology, which became more flattened and elongated (Figure 3).

The number of SA-β-Gal-positive cells among the population of neoplastic cells increased with every passage (Figure 4). All investigated primary cancer cells (prostate, breast, colorectal cancer) were cultured until the majority of cells did not become senescent; however, the number of passages and time of culturing differed among cases, even for the same type of cancer.

Interestingly, when considering senescence occurring in stable cancer cell lines, populations of SA-β-Gal-positive cells were detected in the following: NCI-H460, SK-MEL28, NCI-H1975 and MCF7 (Figure 5C-F). Compared to these lines, no SA-β-Gal-positive cells were detected in MDA-MB-468 and SW962 (Figure 5A and B). The highest proportion of senescent cells in primary cultures was observed for colorectal cancer cells (91%±5.24), and the lowest in breast cancer culture (79%±1.4; colorectal cancer/breast cancer=1.15; p=0.02). Among stable cancer cell lines, SK-MEL28 represented the cell line with the highest number of SA-β-Gal-positive cells culture (36%±2.6), whilst SW962 cell cultures had no SA-β-Gal-positive cells (p=0.03). Comparing cases from both primary cell cultures and stable cell lines with the highest proportion of senescent cells, we observed a difference between those cultures (colorectal cancer/SK-MEL28=2.54; p=0.02) (Figure 6).

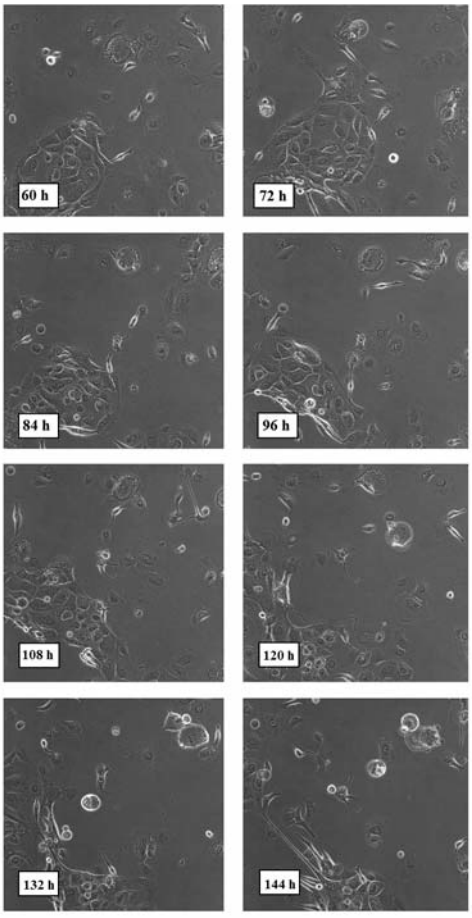

Figure 3. *Real-time phase microscopic observation of primary prostate cancer cells (case no. PC1; passage 2). Observation started after 60 h of culture and was conducted until day 6. Images were taken every 12 h.*

SA-β-Galactosidase and formation of SAHF. According to the literature, SA-β-Gal activity and SAHF formation are two main senescence markers; however, no SAHF-positive cell was detected in any of the samples analyzed, including both primary cell cultures and cancer cell lines, neither in SA-β-Gal-positive cells nor in negative cells (Figure 7).

Discussion

We have already shown that the vast majority of glioblastoma cells can easily become senescent *in vitro* (6,7). Senescence was typically considered to be an antineoplastic phenomenon (1), with special emphasis on oncogene-induced senescence (OIS) as a mechanism protecting against cancer (1). OIS was observed to occur prevalently during pre-malignant changes, and escape from senescence was proposed to be an element of progression to malignancy (14). Most scientists involved in the field of senescence research showed that in cancer cells, this process occurs only after induction by chemical compounds or antitumor drugs (4, 5). Nevertheless, in some articles, a low percentage of SA-β-Gal-positive cells in cancer cell lines was observed in control experiments (15). On rare occasions, authors such as Blagosklonny *et al*. considered cancer cells as pro-senescent, owing to the activation of oncogenes such as *BRAF* or *RAS* (1); however, simultaneous activation of oncogenes and inactivation of tumor-suppressor genes (*TP53, RB*) was shown to be responsible for evasion of senescence. In other words, cancer cells could be conditionally pro-senescent, under conditions of restoring proteins such as TP53 or p16 (16, 17). Several studies confirmed the involvement of senescence in tumor progression, among them Courtois-Cox

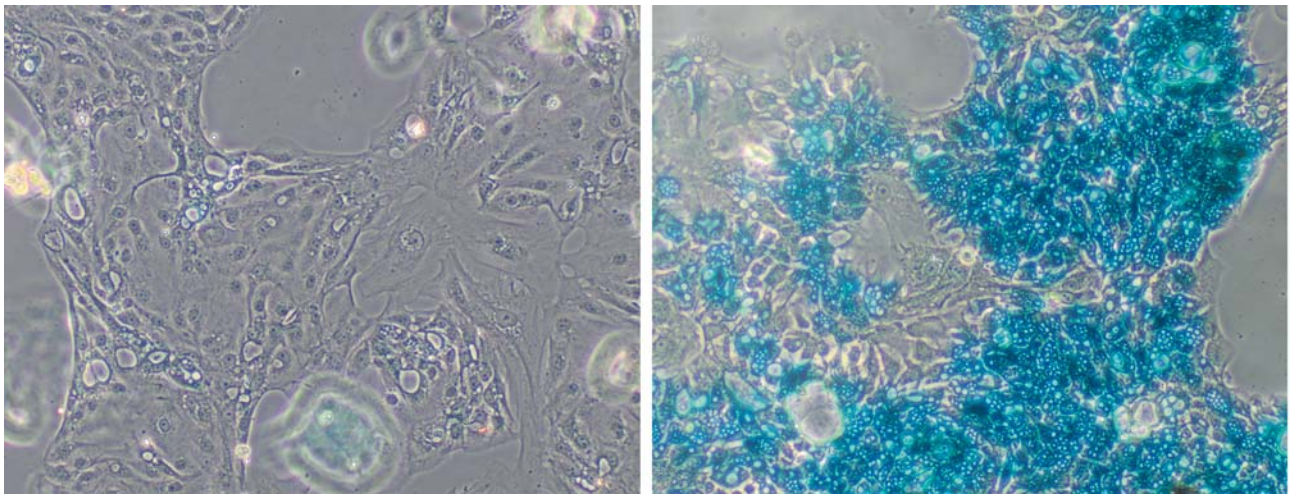

Figure 4. *Results of Senescence-associated-β-galactosidase (SA-β-Gal) analysis in the second (left) and eighth (right) passage of primary breast cancer cells in culture (case no. BC4).*

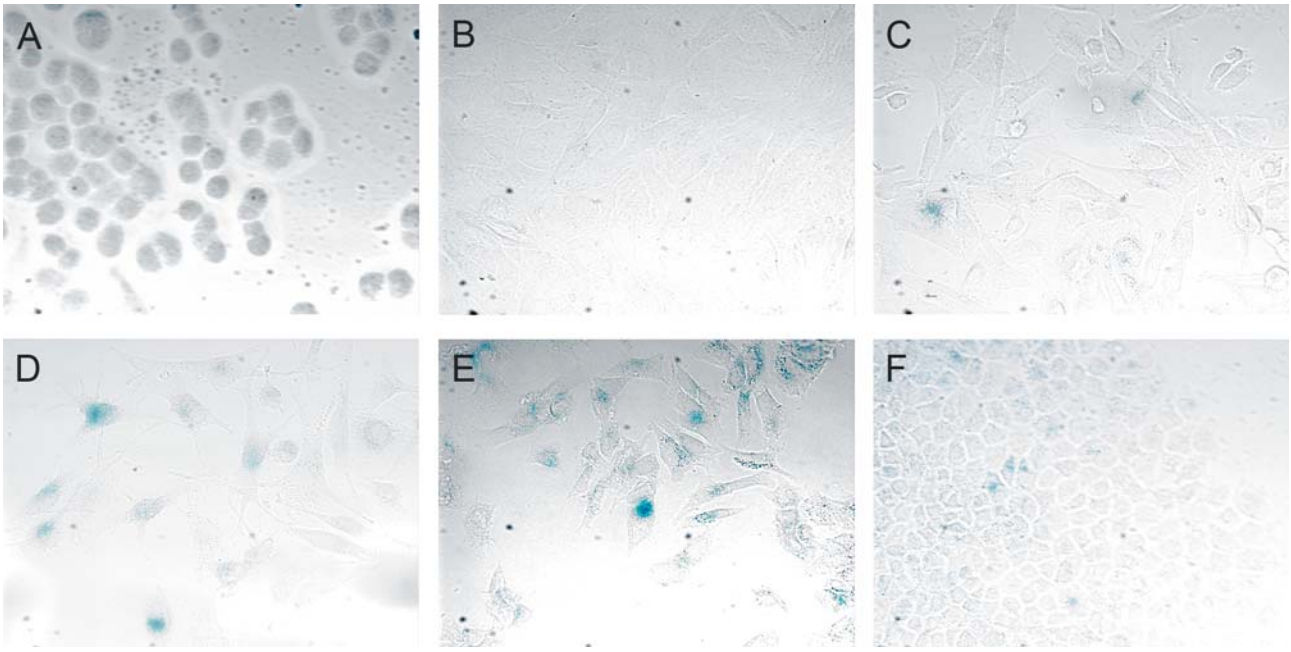

Figure 5. *Senescence analysis of exemplary cancer cell lines. SA-β-Gal-positive cells were not detected in MDA-MB-468 (A) and SW962 (B) cell lines. In contrast, single SA-β-Gal-positive cells were visible in NCI-H1975 (C), SK-MEL28 (D), MCF7 (E) and NCI-H460 (F) cell cultures.*

et al. investigated it in neurofibromas (with a constitutively high level of RAS activity) (18) and Michaloglou *et al*. noticed senescent cells in benign skin lesions (with *BRAF* mutant) (19). Senescence phenotype induced by oncogene activation was observed in many benign lesions such as adenoma; however, no senescence markers were found in adenocarcinoma (malignant lesion) (14).

Our data strongly encourage us to claim that pro-senescence in primary colonic, prostate and breast cancer cells is easily revealed under standard *in vitro* cell culture conditions. We showed that the induction of senescence in primary cancer cells is possible without any chemical or physical stimuli (besides the switch from *in vivo* to standard *in vitro* conditions). The mechanism of cell-cycle inhibition in cancer cells should be recognized in detail. Intriguingly, even cells with *TP53* mutation can become senescent, thus TP53-independent mechanisms must also be present in cancer cells.

An additional issue to be considered when analyzing the mechanism of senescence induction in cancer cells is the lack or presence of SAHF. Some authors suggested that SAHF presence is related to OIS, and lack of these structures could be interpreted in favor of senescence types other than OIS (20). When recognizing SAHF as a marker of OIS, senescence observed under standard cell culture conditions does not fulfill the criteria of OIS. Cancer cells from stable cell lines did not exhibit any SAHF accumulation.

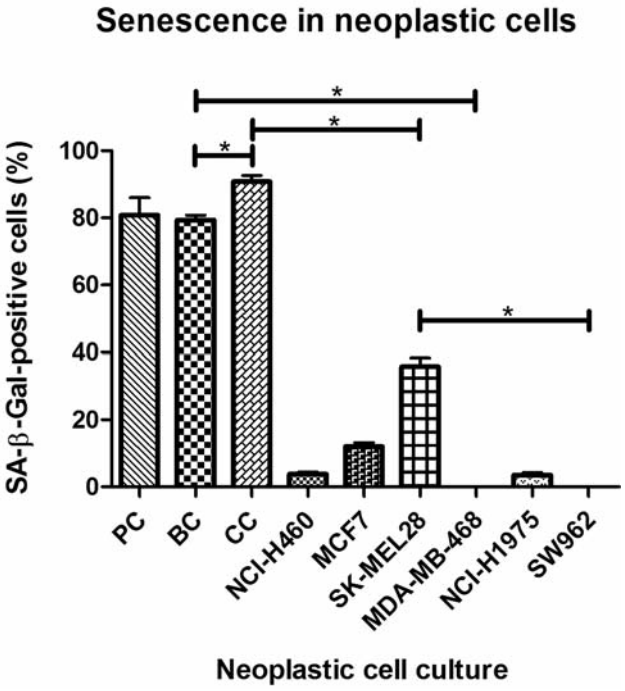

Figure 6. *Comparison of spontaneous senescence in vitro in primary cancer cell cultures and stable cancer cell lines. Differences in percentage number of SA-β-Gal-positive cells between various types of neoplastic cells.*

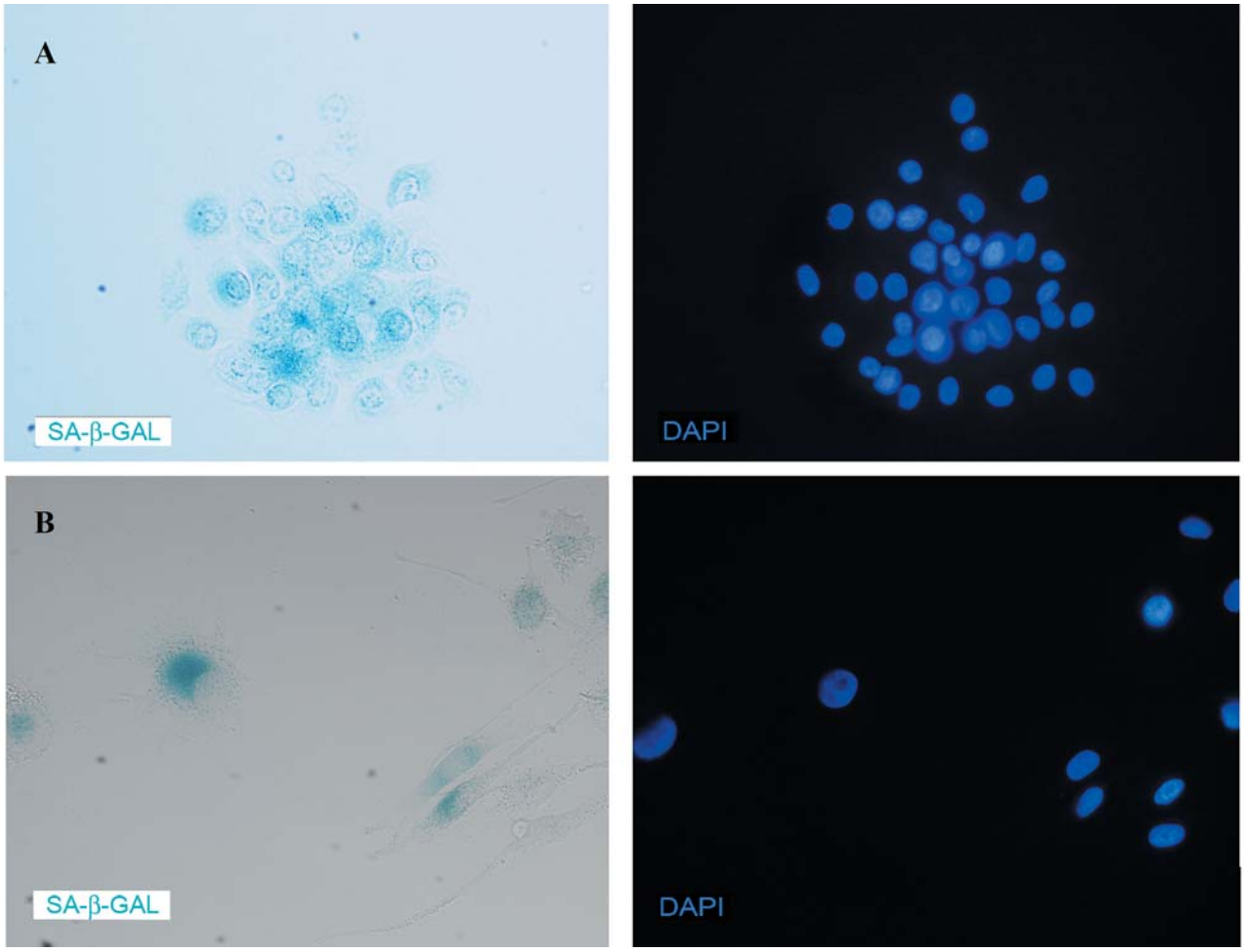

Figure 7. *SA-β-Galactosidase-positive cells did not exhibit formation of senescence-associated heterochromatic foci (SAHF), neither in pimary cancer cell cultures (e.g. prostate cancer, case no. PC1, A) nor in stable cell lines (e.g. SK-MEL28, B). The images on the left and right present the same microscopic field.*

In several cancer cell lines, we found single cells or single colonies of SA-β-Gal-positive cells. An important outcome arising from this study is that stable cancer cell lines represent only a marginal group of cancer cells, comprising cells evading senescence *in vitro*. The majority of cancer cell lines that have been analyzed for many years do not exhibit features now typical of cancer cells, *in vitro* unveiled pro-senescence. In consequence, the general opinion regarding the sensitivity of cancer cells to senescence is strongly biased by the analysis of cell lines which represent only a minority of cancer cells. Obviously, the presence of single senescent cells or even whole senescent colonies in cancer cell lines requires special focus in the future. Apparently, senescence is triggered in many cancer cell lines by standard cell culture conditions. Only the ratio (balance) between senescent and non-senescent cells is extremely shifted in favor of non-senescent ones in cancer cell lines when compared to primary cancer cells.

Until now, senescence of cancer cells in standard cell culture conditions was barely recognized. Our work widens knowledge about the senescence of cancer cells *in vitro*.

Conclusion

In conclusion, pro-senescence in primary prostate, breast and colorectal cancer cells can easily be demonstrated *in vitro* (Figure 6). Moreover, these cancer cells become senescent despite the failure of cell-cycle regulators (such as TP53). Finally, the lack of SAHF formation in SA-β-Gal-positive cancer cells exclude OIS as a type of senescence present in primary prostate, breast and colorectal cancer cells, when recognizing SAHF as a marker of OIS.

Competing Interests

The Authors declare that they have no competing interests.

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References

1 Blagosklonny MV: Cell-cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging. Aging (Albany NY) *4(3)*: 159-165, 2012.

2 Demidenko ZN and Blagosklonny MV: Growth stimulation leads to cellular senescence when the cell cycle is blocked. Cell Cycle *7*: 3355-3361, 2008.

3 Serrano M, Lim AW, McCurrach ME, Beach D and Lowe SW: Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK1A. Cell *88*: 593-602, 1997.

4 Lee YH, Kang BS and Bae YS: Premature senescence in human breast cancer and colon cancer cells by tamoxifen-mediated reactive oxygen species generation. Life Sci *97(2)*: 116-22, 2014.

5 Villalonga-Planells R, Coll-Mulet L, Martínez-Soler F, Castaño E, Acebes JJ, Giménez-Bonafé P, Gil J and Tortosa A: Activation of p53 by nutlin-3a induces apoptosis and cellular senescence in human glioblastoma multiforme. PLoS One *6(4)*: e18588, 2011.

6 Stoczynska-Fidelus E, Piaskowski S, Bienkowski M, Banaszczyk M, Hulas-Bigoszewska K, Winiecka-Klimek M, Radomiak-Zaluska A, Och W, Borowiec M, Zieba J, Treda C and Rieske P: The failure in the stabilization of glioblastoma-derived cell lines: spontaneous *in vitro* senescence as the main culprit. PLoS One *9(1)*: e87136, 2014.

7 Stoczynska-Fidelus E, Och W, Rieske P, Bienkowski M, Banaszczyk M, Winiecka-Klimek M, Zieba J, Janik K, Rosiak K, Treda C, Stawski R, Radomiak-Zaluska A and Piaskowski S: Spontaneous *in vitro* senescence of glioma cells confirmed by an antibody against IDH1R132H. Anticancer Res *34(6)*: 2859-67, 2014.

8 Stoczynska-Fidelus E, Bienkowski M, Pacholczyk M, Winiecka-Klimek M, Banaszczyk M, Zieba J, Bieniek G, Piaskowski S and Rieske P: Different mutational characteristics of TSG in cell lines and surgical specimens. Tumour Biol *35(11)*: 11311-11318, 2014.

9 Park JG, Ku JL, Park SY: Isolation and Culture of Colon Cancer Cell Lines. Methods in Molecular Medicine, vol. 88: Cancer Cell Culture: Methods and Protocols. Edited by S. P. Langdon, Humana Press Inc., Totowa, NJ 79-92, 2003.

10 Pfaffl MW, Horgan GW and Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res *30*: e36, 2002.

11 Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O *et al*: A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. Proc Natl Acad Sci USA *92(20)*: 9363-9367, 1995.

12 Barretina J, Caponigro G, Stransky N, Venkatesan K, Margolin AA, Kim S, Wilson CJ, Lehár J, Kryukov GV, Sonkin D, Reddy A, Liu M, Murray L, Berger MF, Monahan JE, Morais P, Meltzer J, Korejwa A, Jané-Valbuena J, Mapa FA, Thibault J, Bric-Furlong E, Raman P, Shipway A, Engels IH, Cheng J, Yu GK, Yu J, Aspesi P Jr, de Silva M, Jagtap K *et al*: The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity. Nature *483(7391)*: 603-607, 2012.

13 Forbes SA, Beare D, Gunasekaran P, Leung K, Bindal N, Boutselakis H, Ding M, Bamford S, Cole C, Ward S, Kok CY, Jia M, De T, Teague JW, Stratton MR, McDermott U and Campbell PJ: COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucl Acids Res *43(D1)*: D805-D811, 2015.

14 Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguría A, Zaballos A, Flores JM, Barbacid M, Beach D and Serrano M: Tumour biology: senescence in premalignant tumours. Nature *436(7051)*: 642, 2005.

15 te Poele RH, Okorokov AL, Jardine L, Cummings J and Joel SP: DNA damage is able to induce senescence in tumor cells *in vitro* and *in vivo*. Cancer Res *62(6)*: 1876-1883, 2002.

16 Ansieau S, Bastid J, Doreau A, Morel AP, Bouchet BP, Thomas C, Fauvet F, Puisieux I, Doglioni C, Piccinin S, Maestro R, Voeltzel T, Selmi A, Valsesia-Wittmann S, Caron de Fromentel C and Puisieux A: Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. Cancer Cell *14(1)*: 79-89, 2008.

17 Ferbeyre G, de Stanchina E, Lin AW, Querido E, McCurrach ME, Hannon GJ and Lowe SW: Oncogenic ras and p53 cooperate to induce cellular senescence. Mol Cell Biol *22(10)*: 3497-508, 2002.

18 Courtois-Cox L, Genther-Williams SM, Reczek EE *et al*: A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell *10*: 459-472, 2006.

19 Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ and Peeper DS: BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature *436*: 720-724, 2005.

20 Kosar M1, Bartkova J, Hubackova S, Hodny Z, Lukas J and Bartek J: Senescence-associated heterochromatin foci are dispensable for cellular senescence, occur in a cell type- and insult-dependent manner and follow expression of p16(ink4a). Cell Cycle *10(3)*: 457-468, 2011.

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