Secreted protein acidic and rich in cysteine-induced cellular senescence in colorectal cancers in response to irinotecan is mediated by P53

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Cellular senescence is another mechanism that can be exploited to achieve better chemosensitivity and greater tumor regression. Unlike apoptosis, cellular senescence can be induced at much lower concentrations of chemotherapy that are better tolerated by patients. We previously revealed that secreted protein acidic and rich in cysteine (SPARC), a matricellular protein, may function as a modulator of chemotherapy sensitivity by enhancing apoptosis. Here, we examine the effects of SPARC on cellular senescence in the presence of chemotherapy. Cellular senescence is induced only in sensitive colorectal cancer (CRC) cells with low concentrations of irinotecan (CPT-11). However, CPT-11-resistant cells exposed to endogenous or exogenous SPARC can also be triggered into cellular senescence. This induction is associated with higher levels of p16INK4A, p21CIP1, ataxia telangiectasia mutated (ATM) and CHK2. As well, we have previously discovered that resistant CRCs have lower concentrations of SPARC relative to sensitive CRCs (24). Since SPARC is also known to interfere with cell proliferation and cell cycle progression (27), we hypothesize that it may also be involved in promoting cellular senescence following exposure to low concentrations of chemotherapy.

In this study, we investigated the role of cellular senescence as a possible anticancer mechanism is highlighted by (i) the common occurrence of p53 or p16 silencing, bypassing senescent pathways in breast cancer cells (22), (ii) the presence of senescent cells in benign but not malignant tumors and (iii) evidence that lymphomas can engage in a p53/p16INK4A-dependent drug-induced senescence which leads to better prognosis (23). The role of cellular senescence as a possible anticancer mechanism is highlighted by (i) the common occurrence of p53 or p16 silencing, bypassing senescent pathways in breast cancer cells (22), (ii) the presence of senescent cells in benign but not malignant tumors and (iii) evidence that lymphomas can engage in a p53/p16INK4A-dependent drug-induced senescence which leads to better prognosis (23).

Our interest in drug-induced cellular senescence was triggered by recent observations that the matricellular protein, secreted protein acidic and rich in cysteine (SPARC), is involved in chemosensitivity (24, 25) and is associated with improved prognosis in ovarian cancer (26). As well, we have previously discovered that resistant CRCs have lower levels of SPARC relative to sensitive CRCs (24). Since SPARC is also known to interfere with cell proliferation and cell cycle progression (27), we hypothesize that it may also be involved in promoting cellular senescence following exposure to low concentrations of chemotherapy. In this study, we investigated the role of cellular senescence with respect to chemotherapy in both resistant and sensitive CRCs and showed that low doses of CPT-11 were sufficient in producing a senescent response both in vitro and in vivo. Furthermore, we showed that SPARC could increase the effect of CPT-11 in inducing cellular senescence most strikingly in resistant CRCs. This effect appeared to be mediated by a p16- and p53-dependent pathway.

Materials and methods

Cell lines

Human CRC cell lines MIP101, MIP101 cells resistant to CPT-11 (MIP/CPT) and 5FU (MIP/5FU). MIP101 cells overexpressing SPARC (MIP/SP) and empty vector control (MIP/ZEO), RKO and RKO cells resistant to CPT-11 (RKO/CPT) were used. Cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum (NCS) and 1% penicillin-streptomycin and 1% kanamycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO2. For MIP/SP and MIP/ZEO cells, DMEM was supplemented with 10% NCS and 0.1% Zeocin
(Invitrogen). Resistant cells MIP5FU, MIPCPT and RKO/CPT were supplemented with 50 μM 5FU or 5 μM CPT-11, respectively.

Cell synchronization

Cells were synchronized to G0/S phase using a double thymidine block before all in vitro experiments. Cells were blocked for 16 h using blocking solution (2 mM thymidine, 2% NCS in DMEM), released for 14 h with DMEM supplemented with 10% NCS and 1% penicillin-streptomycin and 1% kanamycin (Invitrogen) followed by a second 16 h block.

SA-β-gal assay

Cells were seeded at 10 000–12 000 cells in a 48-well plate for 24 h. Following cell cycle synchronization, cells were treated with 0–20 μM 5-FU or 2–20 μM CPT-11 and 0–10 ng/ml recombinant SPARC protein (rSPARC; kind gifts from Dr Neil Desai; Abraxis Bioscience LLC, Los Angeles, CA). The activity of SPARC was confirmed based on its known interaction with procaspase-8 to enhance apoptosis (25). Cells were washed in phosphate-buffered saline 2× and fixed in 2% formaldehyde and 0.2% glutaraldehyde solution for 15 min at room temperature. Following two washes with PBS, cells were stained with SA-β-gal staining solution [1 mg of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside in dimethylformamide (X-gal), 40 mM citric acid, 40 mM sodium phosphate, 150 mM NaCl, 2 mM MgCl2, 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide, pH 6.0] at 37°C in a humidified chamber for 24 h (5). The percentage of senescent cells, characterized by an intracellular blue insoluble precipitate inside the cell, was visually determined by the total number of positive cells divided by the total cell number. Cells were visualized under ×40 magnification using a Zeiss light microscope and Canon EOS digital camera for image capturing. Four fields of ~100 cells were counted and each experiment was repeated three times.

Cell proliferation, immunoblotting, assessment of apoptosis and reverse transcription–polymerase chain reaction

Please refer to supplementary Materials and Methods (S-methods) for details.

RNA interference

To optimize the conditions for effective knock down of p16INK4A and p53 gene expression with small interfering RNA (siRNA), MIP, MIP/SP and MIP/CPT cells were seeded (six-well plate). Twenty-four hours later, cells were transiently transfected with 20–40 nM of either scramble oligonucleotide sequence (control) or 10 μM of p16INK4A or p53 siRNA (Stealth RNAi; QIAGEN, Valencia, CA) for 72 h using HiPerfect transfection reagent (QIAGEN). Reverse transcription–polymerase chain reaction analysis described above showed that 40 nM of siRNA yielded the most efficient knock down (8-fold decrease for p53 and 13-fold decrease for p16, supplementary Figure D is available at Carcinogenesis Online). For all subsequent experiments, 40 nM of siRNA or scramble control was used. The effects of transient transfections were carried out for at least 72 h. Cells were assessed for cellular senescence using SA-β-gal staining and protein using immunoblotting following p16INK4A and p53 siRNA transfection.

Transient transfection

MIP and MIP/SP cells were seeded at 100 000–150 000 cells per plate on 60 mm plates for 24 h. Following cell cycle synchronization, 0.5 μg of pC53-SN3 vector construct (provided generously by B. Vogelstein (28)), was incubated with 1 μg polyethyleneamine (Sigma, St Louis, MO) in DMEM containing 10% NCS for 2 h and then with complete medium overnight before the addition of 2 μM CPT-11 for another 2 days.

In vivo studies

Tumor xenografts were harvested from National Institutes of Health nude mice (6 weeks old, Taconic Laboratories, Hudson, NY) injected with 2 × 106 MIP101 and MIP/CPT cells as described previously (24). The experimental groups in this study (n = 12 tumors per group, three to four animals per group, each bearing four tumors per animal) included treatment with saline, CPT, SPARC or CPT + SPARC. Upon reaching a tumor size of 100 mm3, animals received 10 μg/kg CPT-11 and 100 μg per mouse rSPARC by intraperitoneal injection three times a week for 3 weeks as described previously (24). At the completion of the study, tumor xenografts were harvested, embedded in optimum cutting temperature compound, sectioned at 6 μm thick, stained and processed for SA-β-gal staining and immunofluorescence as described previously. Primary antibodies used for immunofluorescence were p16INK4A antibody (1:25; Applied Biological Materials, Richmond, BC, Canada) and (p)53 antibody (1:50; Cell Signaling #9284, Danvers, MA). Immunofluorescence was quantified by determining the percentage of positively to negatively stained regions by measuring pixel density of each color channel using Adobe Photoshop 6.0.

Statistics

Statistical difference between experimental groups was calculated and analyzed using Student’s t-test. Statistical significance was defined as P < 0.05.

Results

Low concentrations of CPT-11 induce greater cellular senescence in CRC cells expressing SPARC

Our earlier observations that SPARC can increase chemosensitivity by augmenting apoptosis in response to chemotherapy in addition to slowing cell cycle progression led us to speculate whether SPARC may also play a role in chemotherapy-induced senescence in the presence of lower concentrations of chemotherapies (24). In order to determine whether SPARC expression could contribute to cellular senescence, we began by assessing sensitive CRC cells, MIP101, incubated with low concentrations of both 5-FU and CPT-11. Low concentrations (10–20 μM) of 5-FU had no effect on the proportion of senescent MIP101 cells after a 48 h exposure (Figure 1A). Cell cycle analysis revealed that double thymidine-blocked synchronized cells were completely found in G1 and S phases, whereas ~25% of unsynchronized cells were also found in G2 phase (supplementary Figure C is available at Carcinogenesis Online). Since senescence is a cell cycle-dependent mechanism, we decided to synchronize cells in G1/S phase. We observed that immediately following synchronization, nearly no cells had reached senescence as opposed to 40% after 48 h regardless of drug treatment (supplementary Figure A is available at Carcinogenesis Online). Clearly, the cell cycle did influence the observed percent of senescence and therefore, in all subsequent experiments, cells were synchronized to account for this. When MIP101 and RKO cell lines were exposed to 2 μM CPT-11, the percentage of senescent cells increased significantly in MIP101 (50.7 ± 1.8% versus 43.7 ± 1.2%, P = 0.03) and RKO (16.0 ± 3.1% versus 4.8 ± 1.8%, P = 0.01) relative to untreated controls (Figure 1B). On the other hand, CPT-11-resistant MIP101 (MIP/ CPT) and CPT-11-resistant RKO (RKO/CPT) cells showed no differences in the percent of senescent cells after 2 μM CPT-11 treatment (Figure 1B). However, we observed the effect of 2 μM CPT-11 on our MIP101 cells stably overexpressing SPARC (MIP/SP) and surprisingly found a 2-fold increase in the proportion of senescent cells after being exposed to 2 μM CPT-11 (74.7 ± 3.2% versus 37.3 ± 4.5%, P = 0.002) relative to no treatment (Figure 1B). In turn, MIP/SP cells exhibited higher levels of cellular senescence relative to MIP101 (74.4 ± 3.2% versus 50.7 ± 1.8%, P = 0.003) and drug-resistant MIP/CPT cells (74.4 ± 3.2% versus 27.7 ± 9.0%, P = 0.007) following synchronization and exposure to 2 μM CPT-11 (Figure 1C) which was also 2-fold higher than lower SPARC-expressing cells. Interestingly, resistant CRCs, MIP/SP and RKO/CPT, shown to have significantly lower expression of SPARC relative to their sensitive counterparts (supplementary Figure B is available at Carcinogenesis Online) also responded the least to CPT-11-induced senescence, thereby supporting our hypothesis that SPARC may positively influence cellular senescence.

Exogenous SPARC is capable of increasing cellular senescence in resistant CRC cells

Based on the above results, the proportion of cells undergoing cellular senescence appear to be related to the level of SPARC expression; for example chemotheraphy-resistant MIP/CPT known to have very low SPARC expression failed to achieve greater senescence following exposure to low CPT concentrations. However, MIP/SP cells with endogenous expression of SPARC were capable of significantly increasing cellular senescence in response to CPT-11 in a manner that was significantly greater than medium-low SPARC-expressing control MIP101 cells. Since we previously established that MIP/CPT and RKO/CPT cells had lowest SPARC expression (supplementary Figure B is available at Carcinogenesis Online), we decided to test whether they could be triggered to undergo senescence when exposed to exogenous
To test this hypothesis, we incubated MIP/CPT and RKO/CPT cells with 1–10 ng/ml rSPARC and 2 \( \mu \)M CPT-11 and found that the combination of 10 ng/ml rSPARC with CPT-11 produced a dramatically greater proportion of senescent MIP/CPT (62 ± 2.1% versus 38.7 ± 4.1%, \( P < 0.007 \)) and RKO/CPT cells (14.0 ± 1.0% versus 5.0 ± 1.7%, \( P < 0.003 \)) compared with non-CPT- and non-SPARC-treated cells (Figure 2A and B). While MIP/CPT cells seemed to respond to SPARC and CPT in a dose-dependent manner, the increase in senescence of RKO/CPT cells appeared to be more abrupt, by occurring mainly after incubation with 10 ng/ml of SPARC and CPT-11. In these resistant cell lines, senescence increased up to 3-fold higher when exposed to 10 ng/ml of rSPARC, suggesting that the combination of SPARC and CPT-11 is required for chemotherapy-induced senescence in the face of a chemotherapy-resistant phenotype.

To further evaluate these senescent cell populations, we confirmed the absence of BrdU incorporation in these \( \beta \)-gal-positive cells, whereas \( \beta \)-gal-negative cells continued to replicate (Figure 3A). Specifically, it was noted that after treatment with 2 \( \mu \)M CPT-11, 27.5 ± 1.7% of cells were \( \beta \)-gal positive. However, 22.0 ± 1.7% of cells that had incorporated BrdU were devoid of \( \beta \)-gal staining, whereas just 1.1 ± 0.3% were both \( \beta \)-gal and BrdU positive. In addition, we also measured PAI-SERP1 and 2 expression in our cell lines exposed to CPT-11 because studies have demonstrated upregulation of these genes in senescent cells (6,29). We found a similar increase in the expression of PAI-SERP1 and 2 when MIP/ZEO and MIP/SP cells were exposed to CPT-11 but interestingly not in MIP/CPT (Figure 3B). In addition, we measured caspase-3/7 levels after 48 h of 2 \( \mu \)M CPT-11 exposure and found no changes in any of the MIP-related cell lines (Figure 3C) thereby supporting our observation that 2 \( \mu \)M CPT-11 was insufficient to produce an apoptotic response. These results were further confirmed by terminal deoxynucleotidyl transferase dUTP nick end labeling assay of MIP/SP or resistant MIP/CPT cells exposed to 2 \( \mu \)M CPT-11 (Figure 3D).

Thus far, our results show that cells exposed to higher levels of SPARC in combination with low concentrations of CPT-11 are more probably to undergo cellular senescence. Our next goal was to investigate if any gene known to be involved in cellular senescence may also be contributing to this SPARC-induced effect. A number of genes regulate the cell cycle and specifically in response to DNA damage such as members of the \( p53 \) and \( p16^{INK4A} \) pathways. Interestingly, senescence-susceptible SPARC overexpressing MIP/SP cells had higher expression of upstream \( ATM \) and \( p16^{INK4A} \) when exposed to 2 \( \mu \)M CPT-11 for 48 h compared with untreated cells [Figure 4A, supplementary Figure M is available at Carcinogenesis Online (mean ± SD, \( n = 3 \)). A similar increase in \( ATM \) and \( CHK2 \) was seen in CPT-11-treated MIP/ZEO cells, but not surprisingly, no change in expression of these genes was observed in MIP/CPT cells (Figure 4A).

Western blots provided further evidence of increased \( p16^{INK4A} \) expression in MIP/SP cells. Both untreated and treated MIP/SP cells have higher levels of \( p16^{INK4A} \) protein. As well, MIP/SP cells displayed elevated levels of activated phospho-p53 (serine 15), a site known to be phosphorylated in response to DNA damage, compared...
with MIP101 cells (Figure 4B). Exposure to incremental concentrations of exogenous rSPARC also resulted in a dramatic dose-dependent increase of p16INK4A levels in MIP/CPT cells as well as a smaller effect in RKO/CPT cells. Both MIP/CPT- and RKO/CPT-resistant cells had elevated levels of p16INK4A (Figure 4C), whereas both cell lines also displayed a similar but less dramatic increase in phosphorylation of p53 (serine 15).

Reduction in cellular senescence following knock down of p16INK4A and p53 but the latter only in cells overexpressing SPARC

The p53 pathway is a well-known mediator of DNA-damage induced senescence. Both p53 and p16 pathways converge upon Rb phosphorylation leading to cellular senescence. The relative contributions of each pathway depend on a variety of stresses but a parallel model of activation has been suggested (21). We note that higher levels of p16INK4A are seen in CRC cells overexpressing SPARC, which is the group of cells most probably to undergo CPT-induced senescence. To elucidate a possible mechanism, we used knock down of p16INK4A and p53 by siRNA transfection to investigate their effect on senescence. Pairs of p16INK4A and p53 siRNA were optimized to 40 nM (supplementary Figure D is available at Carcinogenesis Online).

Incubation with 2 μM CPT-11 in conjunction with knocking down p16INK4A resulted in a decrease of cellular senescence across all cell lines: MIP101 by 41.3% (14.7 ± 1.2% versus 25.0 ± 1.3%, \(P = 0.0002\)), MIP/SP by 25.4% (29.3 ± 0.4% versus 39.3 ± 2.1%, \(P = 0.0009\)) and MIP/CPT by 21.9% (20.2 ± 0.5% versus 25.8 ± 2.4%, \(P = 0.04\)) relative to non-specific scramble control.

As expected, MIP/CPT cells exposed to rSPARC had a significantly greater proportion of cells undergoing senescence but this effect was also abolished following p16INK4A knock down. Specifically, MIP/CPT exposed with 10 ng/ml rSPARC had a reduction in senescent cells by 44.8% (18.7 ± 2.4% versus 33.8 ± 2.0%, \(P = 0.001\)).
Fig. 4. Expression of senescence-associated markers is increased in MIP cells exposed to CPT-11. (A) Gene expression of ATM, CHK2 and p16\(^{INK4A}\) increases most in MIP/SP cells when treated with 2 \(\mu\)M CPT-11. Higher levels of SPARC results in elevated phospho-p53 (ser 15) and p16\(^{INK4A}\) protein levels after 48 h when supplied (B) endogenously (assessed by reverse transcription–polymerase chain reaction in MIP/SP cells) and (C) exogenously, following incubation with 1–10 ng/ml of SPARC. Protein lysate of MIP101, MIP/ZEO, MIP/SP, MIP/CPT and RKO/CPT cells were synchronized and exposed to 2 \(\mu\)M CPT-11 and/or 0, 1 and 10 ng/ml recombinant SPARC for 48 h. Full western blots and average densitometries are shown in supplement (supplementary Figures E–K and N–O, respectively, are available at Carcinogenesis Online).

Fig. 5. p16\(^{INK4A}\) knock down eliminates CPT-11-induced senescence in CRCs, whereas knock down of p53 has the same effect but only in cells exposed to SPARC. (A) The percent of senescent cells (MIP101, MIP/SP, MIP/CPT +/− 10 ng/ml SPARC and RKO) increases when incubated with 2 \(\mu\)M CPT-11 (CPT-11 +) for 48 h in combination with non-specific scramble control (p16s −) but transfection with two p16\(^{INK4A}\) siRNA (p16s +) decreases the effect of SPARC and CPT-11-induced senescence as determined by SA-\(\beta\)-gal staining. (B) Knock down of p53 by two siRNA (p53s +) transfection decreases the percent of senescent cells only in MIP/SP and MIP/CPT + 10 ng/mL SPARC but only in cells exposed to higher levels of (either endogenous or exogenous) SPARC. Overexpression of wild-type p53 (wt p53) does not increase cellular senescence in MIP101 (C) but only in cells endogenously overexpressing SPARC (D) relative to untreated, empty vector control cells (pcDNA). The percent of senescent cells were counted in three fields after SA-\(\beta\)-gal staining and represent four experiments. Transfection efficiency of p16s and p53 were shown to be at least 60% as assessed by reverse transcription–polymerase chain reaction (supplementary Figure D is available at Carcinogenesis Online). Statistical difference, asterisks represents \(P < 0.05\) using Student’s t-test.
In a different cell line, such as RKO, a reduction of 48.5% (5.7 ± 0.6% versus 11.0 ± 0.9%, \( P < 0.002 \)) relative to cells transfected with non-specific scramble control treated with CPT-11 (Figure 5A) was also observed with p16 INK4A knock down. Meanwhile, incubation with 2 \( \mu \)M CPT-11 and knock down of p53 reduces the percent of senescent cells only in cells exposed to higher levels of SPARC, either endogenously or exogenously. For example, a decrease by 26.3% (29.0 ± 0.9% versus 39.3 ± 2.1%, \( P = 0.0002 \)) was seen in MIP/SP cells whereas rSPARC-treated MIP/CPT cells had a reduction of 22.7% (26.2 ± 2.7% versus 33.8 ± 2.0%, \( P = 0.02 \)) relative to cells transfected with non-specific scramble control (Figure 5B). Conversely, by introducing overexpression of wild-type p53 in MIP101 cells by transient transfection, the percent of cellular senescence significantly increased but again only in cells overexpressing SPARC (46.6 ± 4.0% versus 31.3 ± 2.6%, \( P = 0.03 \)) relative to cells transiently transfected with pcDNA empty vector control (Figure 5C and D). Interestingly, in MIP/SP cells, transient transfection of p53 alone significantly increased senescence irrespective of the presence of CPT-11 (Figure 5D).

**Fig. 6.** Effect of SPARC and CPT-11-induced senescence in tumor xenografts of CRC cells. Treatment of (A) MIP101 and (B) resistant MIP/CPT tumors with SPARC and CPT-11 increases cellular senescence, p16\(^{INK4A}\) and (p)53 (ser 15) levels. Tumor xenografts of MIP101 and MIP/CPT cells were harvested from mice following saline (control), CPT-11, SPARC or combination treatment and stained for SA-\( \beta \)-gal, p16\(^{INK4A}\) and phospho-p53 (serine 15). Images were taken under halogen (\( \beta \)-gal), DAPI (blue), p16\(^{INK4A}\) (red) or (p)53 (green) (x40 magnification). Tumors exposed to SPARC and CPT-11 (CD) had elevated \( \beta \)-gal (yellow), p16\(^{INK4A}\) (blue) and (p)53 (orange) staining relative to untreated tumors. The percentage area represents five fields across three different xenografts.

In a different cell line, such as RKO, a reduction of 48.5% (5.7 ± 0.6% versus 11.0 ± 0.9%, \( P = 0.002 \)) relative to cells transfected with non-specific scramble control treated with CPT-11 (Figure 5A) was also observed with p16\(^{INK4A}\) knock down. Meanwhile, incubation with 2 \( \mu \)M CPT-11 and knock down of p53 reduces the percent of senescent cells only in cells exposed to higher levels of SPARC, either endogenously or exogenously. For example, a decrease by 26.3% (29.0 ± 0.9% versus 39.3 ± 2.1%, \( P = 0.0002 \)) was seen in MIP/SP cells whereas rSPARC-treated MIP/CPT cells had a reduction of 22.7% (26.2 ± 2.7% versus 33.8 ± 2.0%, \( P = 0.02 \)) relative to cells transfected with non-specific scramble control (Figure 5B). Conversely, by introducing overexpression of wild-type p53 in MIP101 cells by transient transfection, the percent of cellular senescence significantly increased but again only in cells overexpressing SPARC (46.6 ± 4.0% versus 31.3 ± 2.6%, \( P = 0.03 \)) relative to cells transiently transfected with pcDNA empty vector control (Figure 5C and D). Interestingly, in MIP/SP cells, transient transfection of p53 alone significantly increased senescence irrespective of the presence of CPT-11 (Figure 5D).

**CPT-11 and SPARC are capable of reducing tumor growth associated with elevated cellular senescence in vivo**

We have established thus far that MIP101 cells respond to CPT-11 and SPARC in vitro with increased cellular senescence. Next, we investigated whether CPT-11 and SPARC would have the same effect on CRCs in vivo. Tumor xenografts of MIP101 and MIP/CPT cells implanted in nude mice and treated for three cycles (three injections a week) of 10 mg/kg CPT-11 and/or 100 \( \mu \)g of SPARC per mouse were examined. Tumors treated with a combination of CPT-11 and SPARC were smaller overall (716.7 ± 185.7 mm\(^3\) versus 240.4 ± 71.8 mm\(^3\), \( P = 0.05 \) in MIP101 and 573.0 ± 139.6 mm\(^3\) versus 105.9 ± 39.5 mm\(^3\), \( P = 0.003 \) in MIP/CPT). These same xenografts also harbored a large percentage of cells in senescence by \( \beta \)-gal staining. For example, 67% of xenografts of MIP101 tumors from mice treated with CPT-11 positively stained for \( \beta \)-gal compared with 0% with saline (Figure 6A). Amazingly, 100% of xenografts from MIP/CPT tumors from mice that were treated with both CPT-11 and SPARC stained positively for \( \beta \)-gal compared with just 33% in xenografts from mice with saline treatment (Figure 6B). p16\(^{INK4A}\) and phospho-p53 were minimal in xenografts from saline-treated mice, whereas these were elevated in tumor xenografts of mice treated with SPARC and CPT-11 (Figure 6C and D). In particular, regions stained positive for SA-\( \beta \)-gal displayed higher levels of both these proteins (Figure 6). Overall, these results reveal a new biological role for SPARC in enhancing the induction of cellular senescence in tumors exposed to low concentrations of CPT-11.

**Discussion**

Cellular senescence has been repeatedly shown to have a role in cancer cell response to chemotherapies. Low concentrations of the chemotherapy, camptothecin, are able to induce cellular senescence in
HCT116 CRC cells (2). Camptothecin and the analogue CPT-11 are topoisomerase I inhibitors that lead to inhibition of DNA replication and transcription. It is suspected that topoisomerase inhibitors activate the traditional DNA damage response through upregulation of ATM and downstream p53 (30). Independently, p16INK4A has also been shown to become quickly upregulated following CPT-11 exposure in vitro and in vivo (31). Both of these pathways target Rb, preventing the release of E2F transcription factors and initiating cellular senescence. Furthermore, the role of cellular senescence in cancer has been highly suggested to be antitumorigenic. Mice with tumors that are able to enter into drug-induced senescence have significantly longer survival than those with tumors resistant to drug-induced senescence (23). Finally, studies have shown that cellular senescence may act as a back up mechanism to apoptotic failure by providing the cell with tumor suppressive function (23). Our previous work has suggested that SPARC can play a role in resensitizing CRCs leading to tumor regression, which has led us to examine the role of cellular senescence in relation to SPARC.

In this study, we first demonstrate that low concentrations of CPT-11 but not 5-FU are capable of inducing cellular senescence in various CRC cell lines (Figure 1). Interestingly, 5-FU, which causes DNA damage through the incorporation of fluorodeoxyuridine triphosphate into DNA, has been previously found to initiate cellular senescence through a DNA damage response in CRC cells such as HCT116, albeit at significantly higher drug concentrations (>700 μM 5-FU) (32). Currently, no other studies have investigated the effect of low concentrations of 5-FU on MIP101 cells leading to cellular senescence. Though MIP101 cells did not senesce in response to low concentrations of 5-FU, a range of 2–20 μM CPT-11 was capable of significantly increasing cellular senescence in these cells. While higher concentrations of topoisomerase inhibitors have been known to induce apoptosis, low doses have been noted to result in cellular senescence (2). In particular, the case of low drug concentrations that are capable of activating cellular senescent mechanisms in CRCs may be especially notable since drug cytotoxicity remains a serious hurdle in the treatment of CRCs.

An important observation made in this study is that although increased cellular senescence is seen in sensitive CRC cell lines following exposure to low concentrations of CPT-11, the response is diminished in chemotherapy-resistant CRCs. We previously showed that resistant CRCs have lower levels of SPARC (24). However, the presence of SPARC in CRCs has been shown to be resistant to chemotherapy in CRC cells, a delay in G1/S phase has been demonstrated (24), SPARC has been shown previously to regulate cell cycle progression by reducing p53 transcription factor v-Jun modulation of p53 and SPARC function. v-Jun has been observed to down-regulate SPARC in chick embryo fibroblasts by interfering with its proximal promoter region (39,40). Meanwhile, v-Jun has also been shown to stimulate cell cycle progression by reducing p53 transcription activity and p21 expression (41). Nonetheless, the mechanism through which SPARC and p53 may interact would require additional studies.

Our results are highly clinically relevant because DNA damaging chemotherapies, such as SN-38, a metabolite of CPT-11 and VP-16, have been shown to induce senescence in breast cancers but not normal breast tissue from human patients (34). We show that treatment of mice with a combination of SPARC and low dose CPT-11 results in greatly increased senescence compared with lone treatment of high dose CPT-11 in chemoresistant tumors. This increase in senescence is correlated with greater p16INK4A and phospho-p53 (Figure 6). The ability of SPARC to resensitize resistant tumors to cellular senescence suggests that SPARC in combination with standard chemotherapy may indeed be of clinical value. These results are exciting as they shed new insight on a relatively novel anticancer mechanism of SPARC that may be used to exploit cases related to chemotherapy resistance.

Supplementary material

Supplementary Figures A–O and data can be found at http://carcin.oxfordjournals.org/
Funding

Canadian Institutes of Health Research (MOP 82881); Michael Smith Foundation for Health Research.

Acknowledgements

IT Tai is a MSFHR Scholar. We thank Michelle J. Tang and Annie P. Chan for their technical assistance, Dr. B. Vogelstein for the p53 plasmids, and Abraxis Bioscience LLC for rSPARC.

Conflict of Interest Statement: None declared.

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