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REVIEW ARTICLE

To kill tumor cells or permanently paralyze them in senescence?

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Summary

Antitumor agents can inhibit tumor growth by 4 major cellular mechanisms; suppressing proliferation, inducing differentiation, killing the cells or forcing them to senesce. Senescent cells (CS) are in permanent paralysis because they are unable to divide, penetrate the surrounding tissues, metastasize, and respond to treatment. In this short review, we will focus on cellular senescence (CS) induced by retinoids in mammary pre-malignant and tumor cells and its potential clinical implication. Novel information is provided about the role of retinoic acid receptor beta 5 (RARβ5) in mediating the retinoid-induced senescent program.

Key words: breast cancer, retinoid receptors, retinoids, senescence

Cellular senescence as a tumor suppressor mechanism

Replicative senescence is a physiological process typical for normal cells, which is characterized by telomere shortening, permanent growth arrest, specific morphological appearance and deregulation in the function of distinctive genes/proteins [1-2]. In addition to the physiological CS, various cytotoxic agents, irradiation, oxidative stress, some oncogenes (RAS, MYC), tumor suppressor genes, and telomere dysfunction can also induce senescent phenotype in normal and tumor cells [3-5]. This type of CS has been called accelerated or induced senescence. Here, for convenience we will use the expression CS, no matter whether it is spontaneous or induced by antitumor agents. As shown in Figure 1, various antitumor agents can suppress cell and tumor growth generally by 4 independent mechanisms: a) inhibiting proliferation, as consequence of cell cycle arrest (quiescence); b) inducing differentiation, which is usually associated with limited or lack of cell proliferation; c) inducing cell death; and/or d) forcing the cells to senesce. These cellular events may affect not only normal, premalignant and tumor cells, but stroma and endothelial cells as well [5]. Each of the above cellular mechanisms is consequence of multiple and well orchestrated gene alterations which have been recently summarized in several excellent reviews [6-10]. However, no matter how many genes or signaling pathways are up- or down-regulated by antitumor agents, the final outcome at cell and tissue levels are the above 4 cellular mechanisms. When cell differentiation is not terminal, cells may reenter the cell cycle and continue proliferating (Figure 1). Terminally

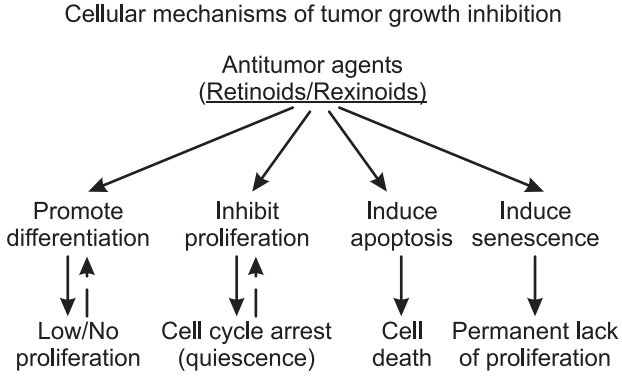

Figure 1. Cellular mechanisms of response to antitumor agents. Solid arrows show how various antitumor agents can suppress tumor growth by promoting cell differentiation, inhibiting proliferation, and inducing apoptosis and/or senescence. Dashed arrows indicate that after removal of antitumor agents, affected cells may return to their normal status of differentiation and proliferation. This is not the case with apoptosis and senescence.

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differentiated cells do not proliferate and usually die by non-apoptotic cell death. Quiescent cells are in transient proliferative arrest and after removal of the antitumor agent may reenter the cell cycle and continue proliferating. Therefore, cell death as consequence of apoptosis, mitotic catastrophe, or necrosis and CS remain the most efficacious mechanisms for permanent tumor growth suppression and eradication [11]. Forcing tumor cells to senesce means permanently inhibiting their capacity to divide, penetrate the surrounding tissue, and/or metastasize, which is equal of permanent paralysis. However, SC may remain for a long time alive and metabolically active secreting various cytokines and thus affecting surrounding and distant cells and tissues [12]. Earlier studies on CS have been predominantly performed *in vitro* on mouse embryo fibroblast (MEFs) and normal cells, which when cultured for a long time (> 20 passages) spontaneously senesce and this has been associated with telomere shortening and/or modulation of p53 – p21 or p16 – pRB signaling [13,14]. However, some cells may overcome the phase in which most cells are senescent (stasis phase), reestablish their proliferative activity and become immortal and later eventually malignant [15]. Tumor cells treated with antitumor agents, radiation and other factors may accumulate specific gene alterations leading to senescence [4,5,16]. Among chemotherapeutic agents used in clinical practice, doxorubicin and cisplatin usually induce DNA damage that may cause CS, whereas taxol and vincristine preferentially target microtubules and affect mitotic division that may lead to development of aneuploid cell clones and gene instability. The genetic background of tumor cells is important for their decision to stop proliferating, senesce, or die by apoptosis or non-apoptotic cell death [17-19]. In previous studies, it was found that p53 is critical for DNA repair induced by radiation or cytotoxic drugs, as well as for development of apoptosis or senescence [13]. In collaboration with Gudkov's group, we reported for the first time that by blocking p53 transcription by a small molecule PIFITRIN in p53+/+ mice, intestinal and spleen cells were protected from radiation–induced apoptosis and senescence [20-22]. This did not happen to p53-/- mice and to mice not protected by PIFITRIN, which did not survive. Since, about 50% of all human malignant tumors, including breast cancer, have mutations in p53, it was suggested that these tumors, when treated with antitumor agents, should not respond by developing senescent phenotype in tumor cells [20]. To prove this hypothesis, patients with p53 wild type and p53 mutant type breast carcinomas have been treated with cyclophosphamide, adriamycin and 5-fluorouracil (CAF) in a neoadjuvant setting and after surgery the percentage of SC have been determined in tumor samples [23]. Senescent cells have been predominantly identified in p53 wild type tumors, confirming the role of p53 in mediating CS in breast cancer. Other molecules that regulate cell cycle progression can also contribute to CS. Among them, $p16^{Ink4a}$, $p21^{Cyp1/waf1}$, and cdk4, which modulate pRB phosphorylation and E2F expression are also involved in mediating CS [24-26]. Treatment of various cell lines that differentially express p21 and p16 with etoposide has been associated with development of CS and overexpression of both genes/proteins [16]. It has been shown that p53 and $p21^{Waf1/Cip1}$ overexpression is mainly associated with initiation of the senescent program, whereas $p16^{INK4a}$ is mostly involved in maintaining the senescent phenotype, therefore p53 and p21 may decrease, whereas p16 increases in SC [4]. In a recent study, we found that dehydroepiandrosterone (DHEA), which is an adrenal 17-ketosteroid precursor to 17β-estradiol and testosterone synthesis and induces lobular differentiation in normal and tumor mammary epithelial cells (MECs), inhibited MNU-induced mammary carcinogenesis in rats by suppressing cell proliferation and inducing CS among

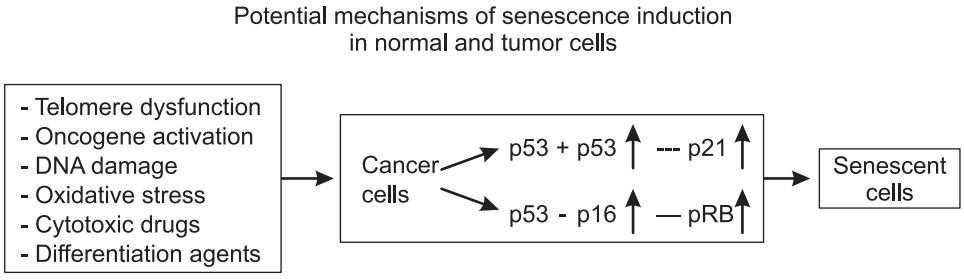

Figure 2. Potential pathways leading to development of senescence in $p53^+$ and $p53^-$ tumor cells. As shown in the left block of the figure, various cellular events may cause CS in tumor cells. In addition to telomere shortening or dysfunction, certain oncogenes (RAS, MYC), agents or factors that induce DNA damage (cytostatics, irradiation), oxidative stress and differentiation agents can also induce CS. In $p53^+$ cells development of CS may affect p21 signaling, whereas in $p53^-$ cells p16-pRB are predominantly modulated.

premalignant lesions and tumors [27]. The increase of SC in mammary tumors was dose-dependent and correlated with increase of p21 and p16, but not of p53 expression, suggesting a p53 independent mechanism of CS. This data indicates that cell differentiation agents, which do not induce DNA breaks, but rather cell differentiation and modulate hormonal environment in mammary gland, can also induce CS. Studies on $p16^{INK1a}$ and cyclin D1 transgenic mice revealed that $p16^{INK1a}$ directly regulates the *in vivo* transition of E2F3 to E2F4 transcription factor, suppresses the formation of E2F complex and thus induces senescence in MECs [14, 25]. In addition to p53 and p16, other cell cycle regulator genes or their modulators may also contribute to the development of CS [28]. Among them, serine/threonine kinase ($BRAF^{V600E}$), insulin-like growth factor binding protein 7 (IGFBP7), chemokine receptor CXCR2, interleukin-8, transcription factor C/ERP, MAPK, ERK and other genes/proteins variably expressed in tumor cells [29,30].

Oncogenes and cellular senescence

Over the last several years intensive research has been done on the role of oncogenes in the development and maintenance of senescence phenotype in normal and tumor cells. Among various oncogenes, MYC and RAS have been mostly examined, and difference in their expression levels has been associated with either promotion of tumor development or with development of senescence [7,8,31,32]. Thus, RAS transfected and overexpressed in MEFs induced cell transformation, but also promoted senescence. We also found that premalignant mammary epithelial cells, MCF10AT, which have been derived from immortal MCF10 cells after stable transfection of Ha-Ras, when treated with inhibitors of farnesyl transferase that suppresses Ras signaling, developed apoptosis, but not senescence [33]. Retinoids, which do not affect Ha-Ras expression, suppress cell proliferation and induce senescence, but not apoptosis suggesting the important role of Ha-Ras in the cell decision to stop proliferating, senesce, or die by apoptosis [34]. In collaboration with Kiyokawa's group, we also showed that cdk4/6, which forms complex with cyclin D1-D3/cdk4/6 and is required for pRB phosphorylation and cell cycle progression, promotes the RAS – induced transformation of MEFs. However, disruption of cdk4 in transgenic mice renders resistance to Ras-induced cell transformation and promotes senescence [35]. The role of RAS in promoting CS was also observed in MNU-induced rats mammary tumors, 50-60% of which carry mutations in codon 12 of Ha-Ras [36,37]. Cells isolated from these tumors and continuously cultured *in vitro* spontaneously senesced after 12-15 passages, whereas those isolated from Ha-Ras negative tumors continued proliferating up to 40-50 passages and did not senesce [27]. In support of this data, the Ghodosh's group recently developed a doxycycline inducible transgenic mouse model that permits RAS activation to be titrated [38]. Low level of RAS stimulates cell proliferation and induces mammary epithelial hyperplasia, whereas high level, similar to that found in tumors bearing K-Ras mutation (lung, pancreas and other types of cancer) induced CS that is $p16^{Ink4a\text{-}Ar\ f}$ - dependent and irreversible following RAS down-regulation. The oncogene-induced CS appears to cooperate with DNA double strand breaks and DNA replication stress involving genes associated with DNA repair [28]. RAS transduced to MEFs activates mitogen-activated protein kinase (MAPK) pathways and induces CS by phosphorylation and activation of extracellular signal regulated (pERK) kinases and $p16^{Ink4a}$ [37]. However, RAS may cooperate with Id1 transcription factor and thus suppress CS and increase metastatic potential of mammary tumor cells [32]. All this data support the hypothesis that genetic background of tumor cells and modulation activity of certain oncogenes and signaling pathways can induce senescent phenotype in tumor cells, which in general are considered immortal.

Molecular determinants of senescent cells

SC can be identified by several methods, among them estimation of senescence-associated - β - galactosidase activity (SA-β-Gal) by histochemistry [39]. This reaction has been considered a strong, although not obligatory biomarker of SC. SA-β-Gal activity is normally detected by using the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-B-D-galactyopyranoside and appears optimal in frozen sections at pH-6.0 [39]. SA-β-Gal increases in SC as consequence of increased lysosomal content. *In vitro,* SC can be identified by their specific morphology: enlarged, flattened cells with vacuolated cytoplasm giving multiple protrusions that make difficult cell borders to be recognized [6,19,40]. In SC the nucleus appears smaller as compared to the cytoplasm, and the nucleus/cytoplasm ratio is smaller as compared to that of normal cells. In SC chromatin is usually condensed in 2-3 large, centrally located particles that can be easily detected by propidium iodide (PI), DAPI or Feulgen staining. The most important feature of SC is their inability to proliferate, they are in permanent proliferative arrest. This can be proven *in vitro* by lack of colony formation or by lack of 5-bromodeoxyuridine (BrdU) accumulation after continuous labeling that

covers at least several cell cycles [41]. To distinguish proliferating from senescent cells, in collaboration with Roninson's group, we labeled cells *in vitro* with a lipophilic fluorescent compound PKH-2 (Sigma) and measured fluorescent intensity at different time points after labeling [41]. PKH-2 labels cell membrane and proportionally decreases in daughter cells after each cell division. Thus, by the level of fluorescent intensity, determined by flow cytometry, one may estimate the number of cell divisions, as well as the proportion of cells that remain undivided. By cell sorter non-dividing cells can be easily separated from dividing and further examined for expression of biomarkers related to senescent phenotype. *In vivo,* and particularly in human tissues and tumors, it is very difficult cells in terminal proliferative arrest to be identified. There are still authors that do not believe SA-β-Gal staining as reliable biomarker of senescence in human tissues. To make sure that retinoids and other antitumor agents can induce senescent phenotype in tumor cells, rats and mice with spontaneous, carcinogen-induced or transplantable tumors were subcutaneously implanted with osmotic pumps that release BrdU for 7 or 14 (2 pumps implanted consecutively) days that correspond to 8-12 or 16-24 cell cycles respectively [42]. This calculation was made based on the information that most xenograft tumors from established breast cancer cell lines and from MNU-induced mammary tumors in rats have cell cycle time (T_C) in the range of 14-18 h [43]. Therefore, cells that remain unlabeled after continuous labeling with BrdU and are positively stained by SA-β-Gal should be considered senescent. In previous studies we also found that SC accumulate lipofuscin, as detected by cytochemistry and that SC are with increased granularity, determined at 90º light scatter by flow cytometry [42]. In most *in vitro* studies and in studies with animal and human tumors treated with antitumor agents or irradiation, overexpression of p53, $p21^{Cip1/Waf1}$ and $p16^{Ink4a}$ have been also reported as indicator of senescence [13,19,24]. However, these biomarkers may also increase in the cell cycle temporary arrested cells (quiescent cells). Therefore, SA-β-Gal staining remains a method of choice for identification of SC both, *in vitro* and *in vivo*. Recently, biomarkers of SC, mostly associated with chromatin condensation (senescence associated heterochromatin foci - SAHFs), senescence associated DNA damage foci (SDFs), DEC1 (differentiated embryo-chondrocyte expressed -1 protein), and DCR2 (decoy death receptor-2) overexpression have been also identified [44]. A good correlation has been found between SA-β-Gal staining and heterochromatin protein 1α and 1γ (HP1α and HP1γ) and trimethylation of Lys 9 of histone H3, suggesting their potential role as biomarkers of SC [28]. However, the specificity and sensitivity of these molecules as biomarkers of SC need further confirmation in various normal and tumor cells and particularly in various tissues and tumors.

Retinoids and induction of cellular senescence

Retinoids and rexinoids have been extensively studied for potential efficacy in prevention and treatment of breast and other types of cancer [45-48]. In addition to inhibition of cell proliferation, they can also induce cell differentiation, apoptosis and/or senescence in normal, premalignant and tumor cells [49]. The advantage of retinoids and rexinoids, as compared to selective estrogen receptor modulators (SERMs) and aromatase inhibitors, is that they suppress the development and progression of both, ER+ and ER- breast carcinomas [50,51]. ER- breast cancer comprises about 30% of all breast carcinomas and over the last 20 years little has been achieved for its prevention and treatment. Most studies, including ours, have shown that retinoids and rexinoids, no matter whether their effect is receptor-dependent or independent, suppress cell proliferation by inducing G1-S and G2-M cell cycle arrest in a dose-dependent manner and in this process cyclin D1, cyclin E, cdks and pRB, but not p53, have been modulated [40,52-54]. Therefore, the retinoid-induced inhibition of cell proliferation and induction of CS have been considered p53-independent. Retinoids can degrade cyclin D1-D3 proteins, inhibit cdk4 activity and thus suppress pRB phosphorylation, E2F activity and cell cycle progression [55,56]. In MNU carcinogenesis model of rats, which simulate many aspects of the development and progression of ER+ human breast cancer, we found that 4-HPR, 9-*cis*-retinoic acid (9cRA), and the rexinoid (LGD1069) induced CS in normal, pre-malignant and malignant MECs [42,57]. The above retinoids were given for 7-21 days to the diet of animals in doses that suppress mammary carcinogenesis. The inhibition of cell proliferation and induction of CS was associated with down-regulation of cyclin D1 and decrease in telomerase activity, both associated with CS [58]. *In vitro* studies with MCF-7 breast tumor cells treated with atRA and 4-HPR have shown that low doses of both agents (100 nM) preferentially suppress cell proliferation and induce CS, whereas higher doses can induce apoptosis [59,60]. Contrary to cytostatics, retinoids and rexinoids at pharmacological doses need time (3-4 days) to suppress cell proliferation and even longer time (7-10 days) to induce senescence. At gene level, atRA and 4-HPR induced alterations in 47 genes (27 were up- and 20 down-regulated) in MCF-7 cells [59]. By quantitative RT-PCR, mRNA of 13 inducible genes was

selected for further analysis: 4 of the retinoid-induced genes were associated with cell division, whereas the other genes were related to cell adhesion molecules and secreted proteins, which SC many release. Among them, TGF-β1, IGFBP-3,-6,-7 and βig-h3 were mostly affected, suggesting involvement of paracrine factors in mediating the retinoid-induced CS. In morphology, SC induced by retinoids appear similar to those induced by genotoxic agents or oncogenes, although they may differ in the gene expression profile and in the signaling pathways involved [60].

Retinoids are ligands of retinoic acid receptors alpha, beta, gamma (RARs, α, β, γ) and can affect normal and tumor cells by modulating transcriptional activity of these receptors [61]. Rexinoids, on the other hand, are ligands of retinoid X receptors alpha, beta, gamma (RXRs, α, β, γ) and may also exert their effect by receptor-dependent and independent mechanisms [62]. The above receptors have been identified in normal and tumor breast epithelial cells, with exception of RARβ and particularly its RARβ2 isoform, which is lost or down-regulated in most breast carcinomas, suggesting a potential tumor suppressor role [63,64]. The lack of RARβ2 in tumor cells is not a result of gene mutation or translocation, but rather of hypermethylation of the gene promoter [65,66]. Dimethylation of the gene promoter may increase the sensitivity of cells to retinoids and improve the clinical response [67]. Transduction of RARβ2 to cells lacking the receptor has been associated with decreased proliferative activity and increased sensitivity to retinoids, as determined by cell proliferation assays, apoptosis, and CS [68,69]. Suppression of RARβ2 transcription may have opposite effect and decreases cell sensitivity to retinoids. Earlier studies from Swisshelm et al. have shown that normal MECs cultured for a long time *in vitro* spontaneously senesce and this has been associated with upregulation of RARβ2 [40]. It was also reported that atRA and 4-HPR at low doses can induce RARβ2 expression in normal, but not in tumor cells and that the low doses preferentially suppress cell proliferation and can induce senescence, whereas high doses (<1.0 μM) may induce apoptosis [49]. In addition to activation of RARβ2, retinoids can also induce proteosome degradation of RARα and thus affect the retinoid receptor signaling [70]. We have recently discovered that short 5´-UTR RARβ2 transcript variants are rare in normal MECs and in MCF-7 cells, but their number increases in ER- breast cancer cell lines, which are considered resistant to retinoids, suggesting that the above transcripts may have negative impact on CS and other mechanisms of cellular response [71]. We also identified a novel isoform of RARβ (β5) (Genbank accession numbers: AC133141.2 and AC098477.2), which appears to play dominant negative role to RARβ2 signaling and is associated with increased resistance of breast premalignant and tumor cells to retinoids [72]. RARβ5 has distinctive promoter P3, which does not have retinoic acid response elements (RARE) and differs from previously known P2 and P1 promoters. The down-regulation of RARβ5 by atRA in MCF10AT cells that carry Ha-Ras let to development of senescent phenotype but not of apoptosis. The same effect was obtained by siRNA, which when transfected to MCF10AT cells suppressed RARβ5 expression and this increased the cell sensitivity to retinoids and to the development of senescence (Figure 3). In a recent study, nude mice with xenografts from early passages of breast cancer have been treated for 4 weeks with atRA. Tumor xenografts expressing RARβ5 were resistant to atRA and did not respond to treatment, whereas xenografts that did not express RARβ5 responded and in addition to inhibition of cell proliferation, developed senescent phenotype [73]. This data indicates that RARβ5 and most probably RARβ2/RARβ5 ratio is important for sensitivity/resistance of breast tumor cells to retinoids.

Potential clinical implications

Cellular senescence, like inhibition of cell proliferation and apoptosis, has also been considered a tumor suppressor mechanism and therefore it might have prognostic and therapeutic implications. Transformation of normal MECs into hyperplastic, premalignant, and malignant cells is associated with progressive decrease in the ability of cells to senesce [4,5,12]. Therefore, breast hyperplastic and premalignant lesions with high percent of SC may have low potential to progress and high potential to regress. There are clinical data generated from consecutive X-ray examination of human breast, indicating that most hyperplastic lesions in pre-menopausal women disintegrate and only few may progress towards premalignant lesions and malignant tumors, supporting the above statement [74]. Since, p53 – p21 and/or p16 – pRB are involved in mediating the senescent program, the lack of their expression in breast carcinomas (mutations in p53 or hypermethylation of p16) may prevent the cells to senesce and these tumors could be considered resistant to treatment. This has been confirmed in a clinical trial where patients with p53 wild and mutated types of breast carcinomas have been treated with neo-adjuvant therapy and SC have been preferentially identified in p53 wild type breast carcinomas [23]. Another alternative are inhibitors of cdks, which are currently used in various preclinical and clinical trials for treatment of breast and other types of cancer. As consequence of cdks inhibition, tumor cells may stop proliferating and/or senesce. Future

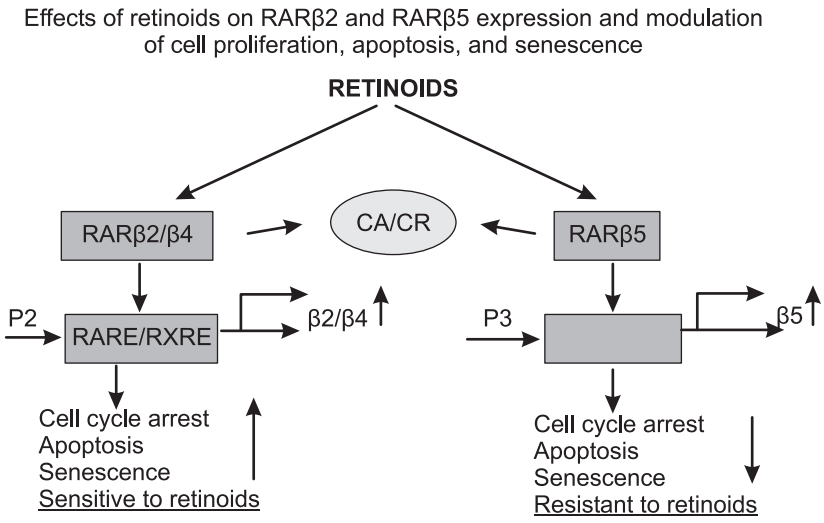

Figure 3. Effects of retinoids on RARβ2 and RARβ5 expression and senescence. Retinoids can induce RARβ2 and RARβ4 isoforms, which apparently cooperate and are regulated by P2 promoter that has retinoic acid response elements (RARE) and retinoid X receptors response elements (RXRE). The up-regulation of both isoforms induces cell cycle arrest, apoptosis, and senescence. Retinoids may also induce RARβ5 isoform, which does not have RARE and RXRE in the promoter (P3) and this is associated with stimulation of cell proliferation, and protection of cells to senesce or die by apoptosis. The biological activity of both isoforms could be modulated by co-activators (CA) or co-repressors (CR).

strategies for treatment of cancer may include development of agents that selectively induce CS and to combine them with other agents that promote apoptosis and thus to achieve maximal clinical effect. SC induced by retinoids in mammary pre-malignant and tumor cells may lead to release of specific cytokines (maspin, IGFBP-3, eplin, FAT10) that affect the surrounding stroma cells and could also be detected in the circulation, and thus serve as biomarkers of response to treatment. Since, the efficacy of retinoids and rexinoids to induce CS depends on the expression level of RARβ2 its up-regulation may increase the potential of cells to senesce. Lost or down-regulation of RARβ2 in breast pre-malignant lesions and carcinomas is consequence of hypermethylation of the gene promoter. Therefore, treatment of patients with dimethylating agents may increase the cell sensitivity to retinoids and the development of senescent phenotype in tumor cells. Since, RARβ2 works in concert with RARα, potential upregulation of the latter by specific ligands may also increase RARβ2 expression and cell sensitivity to retinoids and to development of senescence. Detection of RARβ5 and lack of RARβ2 in breast premalignant lesions and tumors suggest unfavorable prognosis, decreased ability of cells to senesce and resistance to retinoids. Further studies are needed particularly in animal model systems and in human tumors to verify the role of CS as prognostic biomarkers as well as a biomarker of treatment efficacy.

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