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Stress-induced Premature Senescence (SIPS)

–Influence of SIPS on Radiotherapy–

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Stress-induced premature senescence/Cellular senescence/Secretion factor/Stromal cell/Radiotherapy.

Replicative senescence is a fundamental feature in normal human diploid cells and results from dysfunctional telomeres at the Hayflick cell division limit. Ionizing radiation (IR) prematurely induces the same phenotypes as replicative senescence prior to the Hayflick limit. This process is known as stress-induced premature senescence (SIPS). Since the cell cycle is irreversibly arrested in SIPS-induced cells, even if they are stimulated by various growth factors, it is thought that SIPS is a form of cell death, irreversibly eliminating replicating cells. IR-induced-focus formation of DNA repair proteins, a marker of DNA damage, is detected in SIPS as well as replicative senescent cells. Furthermore, both processes persistently induce cell cycle checkpoint mechanisms, indicating DNA damage created by ionizing radiation induces SIPS in normal cells, possibly by the same mechanisms as those occurring in replicative senescence. Interestingly, IR induces SIPS not only in normal cells, but also in tumor cells. Due to the expression of telomerase in tumor cells, telomere-dependent replicative senescence does not occur. However, SIPS is induced under certain conditions after IR exposure. Thus, cell death triggered by IR can be attributed to apoptosis or SIPS in tumor cells. However, metabolic function remains intact in SIPS-induced cancer cells, and recent studies show that senescence eliminate cells undergoing SIPS secrete various kinds of factors outside the cell, changing the microenvironment. Evidence using co-culture systems containing normal senescent stromal cells and epithelial tumor cells show that factors secreted from senescent stroma cells promote the growth of tumor epithelial cells both *in vitro* and *in vivo*. Thus, regulation of factors secreted from SIPS-induced stromal cells, as well as tumor cells, may affect radiotherapy.

INTRODUCTION

Radiotherapy (RT) is one of the best therapeutic choices for cancer treatment. It is the best way to completely remove or reduce the tumor mass for cancer treatment. One of the major topics of radiation biology has been to find more efficient means of increasing the efficacy of ionizing radiation (IR). More recently, increasing the efficiency with which IR induces cell death in tumor *versus* normal cells has been a major focus of research. Since IR-induced apoptosis was reported in 1982,[1] it has been thought that apoptosis plays a primary role in IR-induced cell death and many studies have attempted to address mechanisms of effective induction of apoptosis in RT-targeted tumor epithelial cells.[2,3] Apoptosis is readily induced in tumors derived from haematopoietic, lymphoid, and germ cells. On the other hand, solid tumors derived from epithelial cells show extensive resistance to apoptosis after IR exposure.[2–5] Further research is needed to enhance the apoptotic responses of epithelial cells following IR exposure.

Due to differential sub-lethal damage repair in normal *versus* tumor cells,[6] 2 Gy fractional doses are typically used in conventional RT, and intensity modulated radiation therapy. Either high- or low- dose rates of irradiation are used in brachytherapy. Eventually, both RT regiments give the targeted area a huge total dosage, such as at least 20 Gy.[7] Since normal stromal cells are required for tumor localization, not only tumor cells but also normal cells are exposed to such a dose. Recent accumulated evidence reveal three points: (i) IR induces stress-induced premature senescence (SIPS) instead of apoptosis as a major mode of cell death in normal human fibroblasts;[8] (ii) SIPS is also induced in various kinds of cancer cells;[9] and (iii) SIPS is expressed in a dose-dependent manner, and high doses of IR are more effective in inducing SIPS.[8] These findings tell us that SIPS may

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greatly affect the efficacy of RT, and achievable radiation doses in clinical therapeutic regimen can trigger SIPS-induction in specific human tumor cell lines.[10] In this review, we introduce the mechanism and biological significance of SIPS and discuss the potential influences of SIPS for RT.

BACKGROUND OF SIPS

Originally, SIPS was defined using various cellular stresses, such as ultraviolet radiation (UV), hydrogen peroxide, chemotherapeutic agents, and IR, that caused premature senescence, similar to the phenomenon as replicative senescence.[8,11–17] Sub-cytotoxic doses of these stresses are required to efficiently induce SIPS. Many cell types (*e.g.*, lung, skin, embryonic fibroblasts, melanocytes, endothelial cells, and retinal pigment epithelial cells) undergo SIPS by these treatments. Interestingly, SIPS, but not apoptosis, is the major response in these types of cells *in vitro,* as well as *in vivo*.[8,18] Cells undergoing SIPS share many cellular and molecular features with cells undergoing replicative senescence. The difference between cells undergoing replicative senescence and SIPS appears to be related to the time at which these features are expressed. Replicative senescence is programmed at a specific time when telomeric DNA ends are exposed, while SIPS is not programmed but a response to a given stress. SIPS is not triggered by dysfunctional telomeres. A common feature is that DNA-damage checkpoint machineries have been permanently activated, with irreparable damage at telomere-nonspecific sites serving as a signal to induce SIPS and specific telomeric ends serving as the activating signals in replicative senescence.

Replicative senescence

Replicative senescence is the process that leads to irreversible growth arrest. Hayflick and Moorehead *et. al.* reported replicative senescence in the 1960's. They found that normal human diploid cells proliferate with a finite number of doublings (*i.e.*, the Hayflick limit).[19] Later, the major cause of replicative senescence was found to be telomere instability. The telomere is a complex of telomeric DNA and telomere-repeats binding factors (TRFs) that bind telomere repeat sequences.[20] Telomeric DNA is composed of tandem-repeat DNA sequences, consisting of 6 bases (TTAGGG in humans) and their termini carry 3'-overhang single-stranded telomeric DNA (about 300 bases).[21] This overhang is embedded in a *cis*-duplex telomeric DNA, and as a result, telomere-DNA forms a loop structure (telomere-loop, t-loop) with TRFs.[22] This capping structure inhibits fusion among each telomere and makes telomeres stable. Since telomeres are located at the end of each chromosome, exposed telomere-DNA ends lead to the activation of DNA-damage checkpoint machinery, responses reminiscent of responses to a DNA-double strand break (DSB). However, the natural telomere-end is possibly distinguished from a DSB by capping with t-loops. Initially, more than 10 kb of average telomere length is in human embryonic fibroblasts.[23] However, due to an end replication problem, which is represented by incomplete DNA-replication on either DNA-end, telomeres are gradually eroded with each cell doubling. Eventually, this leads to critical shortening, to nearly 5 kb of average telomere length.[23] When telomere lengths become critically shortened, the average telomere length, as well as their 3'-overhang single stranded telomere length, is shortened,[24] and their t-loop structures are possibly loosened or malformed. Recent knowledge of the molecular stress responses occurring during SIPS or replicative senescence have established that the initial trigger is the sensitive detection of DNA-damage sites, especially DSBs in individual cells. Ionizing radiation-induced foci (IRIF) are protein complexes that include DSB-recognition, mediator, and effecter proteins and are thought to be markers of DSB sites.[25,26] These foci were also observed in replicative senescent cell populations with shortened telomeres and co-localization with TRFs was demonstrated at telomere ends.[27,28] These data strongly suggested that shortened telomeres induce telomere-dysfunction, exposing telomere-ends and signaling DSB-mediated stress responses.

Following the appearance of dysfunctional telomeres, the *ataxia telangiectasia* mutated (ATM)-p53 pathway is activated and greatly contributes to irreversible growth arrest, which is a major feature of replicative senescence. In response to DSBs, inactivated forms of dimeric or multimeric ATM complexes are dissociated to active monomers, that are eventually autophosphorylated at Ser1981.[29] In fact, phosphorylated ATM is involved in foci located at telomeric ends[27] (called telomere dysfunction-induced foci, TIF, in case of replicative senescence. Nearly all the components of TIF are involved in IRIF.) This active form of ATM, in turn, phosphorylates various ATM targets. In the case of p53, activated ATM directly or indirectly phosphorylates serine/threonine residues within the N-terminus of p53.[30] As a result, cells in this period stabilize p53, which in turn acts to transactivate a number of downstream genes. One of the most important p53 downstream genes is p21.[31] p21 acts to inhibit cyclin-dependent kinase 2 (CDK2)[32] and blocks functional CDK2/ cyclin E complexes, inhibiting the progression of cells from G_1- to S-phase of cell cycle. As a result, cells are permanently arrested in the G_1-phase of the cell cycle. Meanwhile, expression of telomerase, which is a reverse transcriptase acting at telomeric DNA sequences, maintain telomere lengths and ultimately delay ATM stress responses, and p53 activation. The result is an extended the Hayflick limit, strongly suggesting that dysfunctional telomere-induced activation of the ATM-p53 pathway plays a critical role in irreversible growth arrest and maintaining a replicative senescence phenotype.

Induced p16 levels represents another example of a simul-

taneous regulatory pathway to maintain cells in an arrested state.[33] p16 inhibits cyclin-dependent kinases 4 and 6 (CDK-4 and -6), which are associated with progression of G_1 cells into S-phase and play a different but redundant role in G_1-arrest response during senescence.[34] Regulation of p16 is accomplished by the hyperactivation of the oncogenic Ras pathway during oncogenic Ras-induced premature senescence,[35] suggesting that p16 induction results from non-telomeric signals. It remains unclear as to the mechanism of p16 induction in senescent cells, however, several molecules like Ets-1, Bmi-1, and Id-1 have been implicated in its expression.[35,36] Eventually, two different pathways, the ATM-p53-p21 and p16 checkpoint modules, appear to be regulated by a dysfunctional telomeric signal and a non-telomeric signal, respectively. There redundantly maintain irreversible growth arrest in senescent cells.

Senescent cells show different features from proliferative cells.[19] There are senescence-specific features, observed as morphological changes and specific gene expression patterns that define senescence. A typical senescence-specific morphology appears as 'flattened and enlarged' cell phenotypes. Age-related gene expression changes have been reported, and some of these are used as "markers" of senescence. Senescence-associated β-galactosidase (SA-β-gal) activity is commonly used as a senescent marker. Since this enzyme works under acidic pH conditions in a senescence-specific manner, senescent cells can be recognized as positive cell by this assay.[37] The significance of a lowered pH in senescent cells has not been explored, but may represent altered cellular metabolism, such as enhanced glucose utilization, in these cells. Clusterin/Apolipoprotein J/x-ray-induced protein-8 (xip8), fibronectin, osteonectin, and SM22 are also known molecular markers of senescence at this point.[38]

SIPS by IR in normal human cells

Cells undergoing SIPS show senescence-like growth arrest (SLGA), which represents irreversible growth arrest with features of senescence-specific cell morphology and gene expression,[39] and one of the phenomena shown in SIPS-induced cells. SLGA appears to be induced by similar mechanisms found in replicative senescent cells. For example, p53 stabilization and transactivation, as well as the p16 pathways appear to maintain irreversible growth arrest. IR-treatment creates DSBs, resulting in ATM-p53-p21 pathway activation and p21-mediated G_1 growth arrest. p53 accumulation and phosphorylation at Ser15 occurs within 2–4 hr after IR.[40] Normally, in cells that recover from this initial insult, p53 responses wane within 6 hr post-IR. However, in SIPS cells, these responses have been continuously observed at least for 10 days.[8] p16 shows delayed expression compared to the induced p53 pathway. p16 induction is observed ≥ 5 days after treatment.[8] SA-β-gal staining appears from 3 days after exposure to ≥ 4 Gy of X-rays, and nearly all the remaining irradiated cells show SA-β-gal-positive staining 5 days after treatment.[8] These responses are observed in a dose-dependent manner.

These observations highlight the fact that IR-induced DNA damage persists in cells undergoing SIPS. IRIF-formation peaks within 30 min in irradiated cells, followed by their decay in two steps: (i) the first decay rate of IRIF disappearance is rapid and within 4 hr after IR exposure most foci disappear; (ii) However, during the second decay rate a much slower reduction in the kinetics is apparrent.[41] This two-step decay rate is directly related to the kinetics of DSB repair.[42] Many IRIF disappear within 1 day after irradiation, however, a few IRIF are still observed in each irradiated cell after high dose IR exposures, and these foci persist for at least 5 days post-treatment.[41] p53 immunostaining data show that p53 accumulates in the nuclei of cells with substantial IRIF. Interestingly, p53 also aggregates around the site of IRIF. The phosphorylated form of p53 at Ser15 is, particularly, detected at IRIF, suggesting that p53 is directly activated by IR-induced damage during SIPS-induction.[41] Apoptotic nucleosomic DNA ladder formation is not observed during SLGA-expression.[8] Thus, cells appear to decide between replicative senescence and apoptosis during this crisis period, with epithelial cells commonly choosing replicative senescence over apoptosis and other cell types (*e.g.*, lymphocytes) more pre-disposed to apoptotic cell elimation. The intracellular regulatory mechanisms that control cellular senescence over apoptosis or necrosis are not completely known.

Telomere instability is not associated with SIPS induction. Since dysfunctional telomeres cause replicative senescence, it was initially thought that dysfunctional telomere origins induce SLGA responses. However, telomeric DNA extracted from cells undergoing SIPS did not show the telomeric shortening, in contrast to length-shortening observed in non-irradiated proliferating cells undergoing replicative senescence.[8] Interestingly, IR does not appear to accelerate telomere erosion. In contrast, Smogorzewska *et al.* showed that the mechanism of telomere instability occurred in a telomere length-independent manner.[43] TRF2 plays a crucial role in t-loop formation. TRF2 forms homo-dimeric complexes that bind to telomeric DNA via their Myb-domain in C-terminus of TRF2.[44] When a dominant-negative form of TRF2 ($TRF2^{\Delta B \Delta M}$), which lacks a DNA-binding domain and suppresses the function of wild type TRF2, was overexpressed, dysfunctional telomeres appeared without telomere-shortening. In addition, transfection of $TRF2^{\Delta B \Delta M}$ also induced SLGA by activating the ATM-p53 pathway.[44,45] This finding suggested that telomere-shortening was not a sole pathway to induce dysfunctional telomeres in cells, although this manipulation of cells was completely artificial since expression of this dominant-negative TRF2 does not exist in nature. Immunofluorescence *in situ* hybridization (Immuno-FISH), that combines

methods with telomere FISH and immunostaining to co-localize telomere ends with selected targeted proteins, shows that IRIF in SIPS-induced cells localizes at different sites from telomere ends.[41] Furthermore, expression of hTERT (human telomerase reverse transcriptase) did not prevent SA-β-gal expression in different types of normal human diploid cells undergoing SIPS by IR, as well as UV and hydrogen peroxide exposure.[46,47] Thus, a few, persistent and irreparable DNA-damaged sites are sufficient to induce SLGA in a telomere-independent manner and they are mediated by the ATM-p53 pathway.

SIPS in cancer cells

Due to telomerase expression in more than 90% of cancer cells,[48] telomeres are typically stable and replicative senescence is not usually induced in cancer cells. However, many agents used to treat tumors, for example, IR[49] or doxorubicin,[50] cisplatin,[51] and camptothecin[52] by chemotherapy, can induce SIPS even in cancer cells. In contrast to replicative senescence, such drug treatments do not affect telomere lengths.[53] Instead, these treatments produce DSBs, and a common cause for SIPS induction in cancer cells appears to be irreparable DNA breaks. Furthermore, cell fusions between tumor and normal cells, or expression of p53, p21, or p16 in tumor cells resulted in induction of senescent phenotypes in tumor cells.[54–56] It is possible that the lack of proper regulation by cell cycle checkpoint machinery loses SIPS-induction in cancer cells. In addition, SIPS induction in p53- or p21-null HCT116 human colorectal carcinoma cells was suppressed compared to parental wild-type HCT116 cells after treatment with SN-38, a DNA topoisomerase I inhibitor.[50] Furthermore, SIPS was noted in tumor tissue samples after chemotherapy treatments and these responses correlated with high levels of p16 expression.[50] Collectively, all evidence indicates that SIPS in cancer cells is induced through the same pathway as replicative senescence. The two processes, however, differ in the triggering mechanisms that stimulate each phenomenon.

BYSTANDER EFFECT FROM SENESCENT STROMA CELL TO CANCER CELL

Co-culture of cancer cells with normal human replicative senescence or SIPS alters tumor cell growth

Processes involved in senescence have usually been performed using normal human fibroblasts. Yet, most cancers are not derived from fibroblast origins. Question arises as to whether the formation of replicative senescence or SIPS in fibroblast populations (*i.e.*, stromal cells) affects tumor growth. One of the ideas for this question is the bystander effect from senescent stroma cell to cancer cell. Bystander effect in radiation biology was established as the mechanism that irradiated cells affect to unirradiated cells via the gap junction intercellular communication (GJIC) or secretion proteins.[57] Since DNA damage has occurred in senescent cells, these cells have the potential to affect other cells around senescent cells. Fibroblasts are most common in connective tissue and provide many factors that constitute the "microenvironment" by synthesis, and maintenance of the structural framework, known as the stroma. To function as stromal cells, fibroblasts continuously secrete components of the extracellular matrix.[58] Also, fibroblasts secret growth factors and cytokines that affect nearby epithelial cells.[58] Co-culture of pre-neoplastic or malignant epithelial cells with senescent fibroblasts accelerates cell growth of both epithelial cells. In contrast, co-culture of normal epithelial cells with senescent fibroblasts does not accelerate growth, at least in one recent study.[59] This group also examined how senescent cells affected cell growth, and found that at least 50% of acceleration was derived from a secreted factor originating from senescent fibroblasts.[59] Meanwhile, co-culture of tumor epithelial cells with the immortalized stromal cells by telomerase expression did not affect to tumor epithelial cell, suggesting that senescent fibroblasts-specific secretion factors affected to pre-neoplastic and malignant epithelial cells.

Senescent cells undergoing SIPS also affected the microenvironment, allowing epithelial cancer cells to accelerate their growth. Fractionated exposure to low-dose IR induced SIPS in normal human mammary fibroblasts, and co-culturing these with breast cancer cells showed accelerated growth of breast cancer cells compared with growth in co-culture with non-senescent cells.[60] Thus, replicative senescence and SIPS-induced a growth advantage to cancer epithelial cells under co-culture conditions both *in vitro,* as well as *in vivo*.

Secreted proteins from senescent cell

Senescent cells show specific phenotypes and loose the capacity to proliferate, yet these cells remain metabolically alive. An important feature of fibroblasts undergoing replicative senescence or SIPS is that these senescent cells remain metabolically active and secrete various factors. Some factors maintain the stroma or stimulate growth of epithelial cells, however, senescent cells also show different secretion patterns or expression levels of growth factors, cytokines, and extracellular matrix proteins as compared to actively replicating fibroblasts.[60,61] Interestingly, the downstream affects caused by secreted factors from senescent cells depends on the recipient cell type, and also secretion pattern depends on stromal cell types.[58] In case of SIPS, various senescent cancer cells may secreted tumor-growth promoting proteins (*i.e.*, TGFα, angiogenic factor cyr61, and the anti-apoptotic factors, Galectin-3 and Prosaposin), as well as tumor-growth suppressing factors (*i.e.*, IGFBP-3, 4, and 6 and MIC1).[62–65] Depending on the localization of the tumor, accumulation of senescent cells by aging or therapies (*e.g.*, RT) may have profound affects on the survival

responses of cancer cells.

CONCLUSION: ROLE OF SIPS FORMATION IN RADIOTHERAPY

It has been theorized that cellular senescence functions as tumor suppressor mechanisms to prevent the growth of cells at risk for neoplastic transformation. Senescent cells with DNA damage created by dysfunctional telomeres or by therapeutic exposure would be permanently restricted in their abilities to proliferate, leading to an alternative cell death thereby playing a role suppressing tumor formation.[66,67] It is now understood that senescence (by replicative senescence or SIPS) is a more general reaction of cells to a wide range of irreparable DNA breaks, and SIPS may also serve as a tumor suppressor mechanism.[27,29,30] One experiment *in vivo* supports this hypothesis. Tyner *et al.* generated p53+/m mice that have a normal copy of the p53 gene and a mutant copy.[68] This mutant gene is deleted from exons 1 to 6 of p53 gene and encodes a truncate form of protein that supports the stability and transcriptionally active of wild type p53 by a mechanism unknown. As a result of activated p53, these mice are resistant to tumor formation, while at the same time, these animals prematurely age. Since p53 deficiency increases cancer incidence, p53 suppresses tumorigenesis via the senescence mechanism not only *in vitro* but also *in vivo*.

Along with responses occurring in normal cells, SIPS can be induced by IR in tumor cells derived from epithelial origin. In general, apoptosis can be induced by IR in tumor cells derived from haematopoietic, lymphoid, and germ cell,[2] however, other solid tumors derived from epithelial cells are commonly resistant to apoptosis, but responsive to SIPS-induced loss of replication. High dose of IR efficiently induce SIPS and the total dose used by RT is enough to induce this process.[7] A recent study *in vitro* with fractionated IR exposures shows SIPS-induction, even if each dosage was below individual doses used by RT.[60] These data suggest that SIPS may contribute clinically to the effects induced by RT. The advent of radiotherapy using proton or heavy ion beam irradiations will inevitably lead to increases in SLGA-induction *in vitro*.[69] Thus, the contribution of SIPS will need to be evaluated as a therapeutic effect of RT, especially in tumors derived from epithelial cell origins-basically a majority of neoplasms.

Importantly, co-culture studies using senescent cells and nontreated tumor cells show that senescent cells confer a growth advantage to tumors. Furthermore, tumors subjected to RT also survived better, that is, the presence of senescent stromal cells appears to provide a pro-survival microenvironment that accelerated tumor growth.[59,60] It was estimated that at least 50% of tumor growth acceleration was caused by secretion factors from senescent stromal cells. Since senescent stroma cells secrete (i) growth factors and cytokines, (ii) extracellular matrix proteins, and (iii) anti-apoptotic factors, the role of these pro-survival factors individually need to be evaluated for their influence on radiosensitivity.

The benefit of RT is its ability to treat non-invasive cancers that are not amenable to surgery, and may be only partially treated with chemotherapy. To make the best use of these benefits, we will need to increase the selective radiosensitivity of cancer cells. We now realize that SIPS is one of the therapeutic byproducts of RT, and that induction of apoptosis in solid tumors is a rather rare and possibly non-meaningful event. However, we are also facing the Yin and Yang of SIPS effects, that is, we can prevent tumor growth by inducing SLGA in tumor cells, and therefore effectively

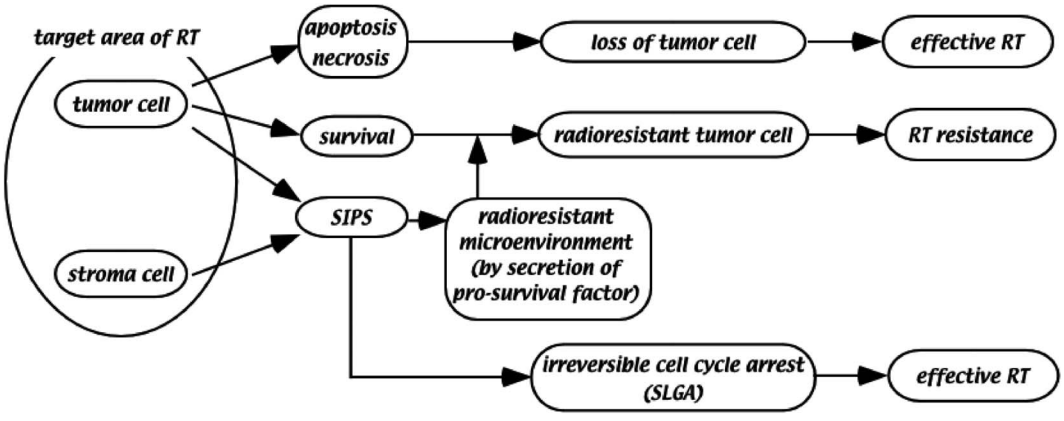

Fig. 1. A possible role of SIPS in RT. Tumor cells undergoing SIPS may contribute to two opposite functions through phenotypes, SLGA or secretion of pro-survival factors. SLGA suppresses tumor growth by the induction of irreversible growth arrest in tumor cells, as a result, contribute to positive effect by RT. In the meanwhile, SIPS-induced cells by initial RT in tumor cells as well as in stroma cells provide radioresistant microenvironment by the secretion of pro-survival factors, as a result, SIPS induced cells protect survival tumor cells from following RT and acts as negative factor against RT.

blocking the replicative nature of the cell. However, we are at the same time providing an environment that promotes radioresistance through the induction of secreted proteins, such as clusterin, in the normal human stromal, as well as in RT-targeted tumor epithelial cells. It is, therefore, essential to better understand the molecular mechanism and the regulation of these secreted factors. All together, it will be eventually possible to suppress tumor cell growth and further increase the radiosensitivity of tumor cells (Fig. 1).

Cancer is an age-related disease and tissue samples from elderly people show that senescent cell accumulate with age *in vivo*.[70] Since senescent fibroblasts, caused by replicative senescence, also secrete proteins and thereby create a radioresistant microenvironment, we must also understand the role of these secreted factors and their contribution to carcinogenesis in the elderly. Furthermore, understanding the signal transduction processes that regulate these secreted proteins, and learning how to prevent their expression, may be required to further improve cancer therapy with RT, as well as chemotherapies. The role of accumulated SIPS and/or replicative senescence in older individuals, as well as after repeated therapeutic regimen in young, as well as older, patients may be a very important resistance mechanism operating in cancer therapy. Understanding the upstream signaling mechanisms, as well as the downstream protein functions will be important for improving therapies against cancer.

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