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Review

REVIEW

Disruptive chemicals, senescence and immortality

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Abstract

Carcinogenesis is thought to be a multistep process, with clonal evolution playing a central role in the process. Clonal evolution involves the repeated 'selection and succession' of rare variant cells that acquire a growth advantage over the remaining cell population through the acquisition of 'driver mutations' enabling a selective advantage in a particular micro-environment. Clonal selection is the driving force behind tumorigenesis and possesses three basic requirements: (i) effective competitive proliferation of the variant clone when compared with its neighboring cells, (ii) acquisition of an indefinite capacity for self-renewal, and (iii) establishment of sufficiently high levels of genetic and epigenetic variability to permit the emergence of rare variants. However, several questions regarding the process of clonal evolution remain. Which cellular processes initiate carcinogenesis in the first place? To what extent are environmental carcinogens responsible for the initiation of clonal evolution? What are the roles of genotoxic and non-genotoxic carcinogens in carcinogenesis?

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What are the underlying mechanisms responsible for chemical carcinogen-induced cellular immortality? Here, we explore the possible mechanisms of cellular immortalization, the contribution of immortalization to tumorigenesis and the mechanisms by which chemical carcinogens may contribute to these processes.

Abbreviations

BaP	benzo(a)pyrene
CDK	cyclin-dependent kinase
CoQ10	coenzyme Q10
GC	genotoxic carcinogen
MEF	mouse embryo fibroblasts
NAC	N-acetyl-cysteine
NGC	non-genotoxic carcinogen
OIS	oncogene-induced senescence
PB	phenobarbital
ROS	reactive oxygen species
SAHF	senescence-associated heterochromatic foci
SASP	senescence-associated secretory phenotype
SHD	Syrian hamster dermal cell
SOD	superoxide dismutase.

An introduction to cellular senescence

Since the early 1980s by seminal works of Newbold *et al.* (1,2) it is known that cellular senescence is a barrier to tumorigenesis. Recent genetic experiments have contributed to explain why oncogenic signals need to bypass this barrier to induce tumors. Therefore, carcinogens, to promote tumorigenesis, must bypass this senescence barrier (1). But, what is this barrier and how it can block the process of carcinogenesis?

In continuous culture, somatic cells show a spontaneous decline in growth rate that is unrelated to the amount of time elapsed during culture; however, this decline is related to a decreasing number of population doublings. Somatic cell aging eventually terminates in a quiescent but viable state termed replicative senescence (3). Cells in this state exhibit specific features and this behavior is observed in a wide variety of normal cells (4). Furthermore, it is widely accepted that normal human somatic cells, with the exception of stem cells and tumor cells, have an intrinsically limited proliferative lifespan, even under ideal growth conditions. Cells that display characteristics of senescence are also observed in response to other internal or external stimuli, such as oncogenic stress, DNA damage or cytotoxic drugs (5).

Characteristic senescent features include flat morphology in culture, multinucleation and a terminal arrest resulting in increased levels of many cell cycle inhibitors. Moreover, the senescent phenotype is associated with dramatic changes in gene-expression (6–9). Senescent cells show altered lysosome/vacuole function and accumulation of mitochondrial damage, which lowers adenosine triphosphate production and increases reactive oxygen species (ROS). Furthermore, enzymes and lipids are damaged by secondary chemical modifications, such as oxidation, glycation or cross-linking, accumulating in the cytosol and lowering the rate of essential cellular functions (10–12).

The onset of senescence triggers the generation and accumulation of distinct heterochromatic structures known as senescence-associated heterochromatic foci (SAHF) (13), which provide an explanation for the stability of the senescent state. Senescent cells also show altered DNA methylation processes (14,15) and display molecular characteristics of DNA damage (16–18), including nuclear foci of phosphorylated histone H2AX and DNA-damage checkpoint factors, such as 53BP1, MDC1 and NBS1 (9,16). Senescent cells also contain activated forms of the DNA-damage checkpoint kinases Chk1 and Chk2. These and other markers suggest that telomere shortening initiates senescence through a DNA damage response. These characteristics also explain why other DNA damage stressors, such as culture shock, can potentially initiate senescence without telomere involvement (11,19).

The finite number of divisions during replicative senescence, which is commonly known as the 'Hayflick limit', is attributed to the progressive shortening of chromosome ends containing the telomeres, which is the proposed molecular mechanism of a senescence clock (20). Eukaryotic cells cannot replicate the distal ends of their telomeres, which shorten in length with every cell division until they reach a critical threshold at which cells stop dividing (21,22).

As mentioned previously, cellular senescence can be elicited by other types of stress, including oncogene activation (23). This phenomenon is observed with many but not all oncogenes, including RAS and its effectors RAF, MEK and BRAF as well as PI3K or AKT (24–27). Also, the activation of other proliferative genes such as CDC6, cyclin E and Signal transducer and activator of transcription 5 (STAT5), or the loss of tumor suppressor genes, such as PTEN, Spn or NF1, can trigger a DNA damage response and induce senescence (28,29). This response is associated with DNA hyper-replication and seems to be the cause of oncogene-induced senescence (OIS) *in vitro* (30–33). Thus, oncogene- or stress-induced senescence does not rely on telomere shortening (34,35). Stress-induced premature senescence shares some of the morphological and biochemical features of replicative senescence activated by telomere shortening (36–40), supporting the hypothesis that senescence is a common response to cellular damage (41).

Recently, a physiological role for senescence in embryonic development has been also uncovered (42,43). This function seems to be dependent on the cyclin-dependent kinase (CDK) inhibitors p21CIP1 and p15INK4b but independent of other cell cycle inhibitors, DNA damage or p53. This senescence during embryonic development is regulated by the PI3K/FOXO and TGFb/SMAD pathways (42,43).

Senescence and immortalization: two sides of the same coin

Immortalization can be defined as the process by which cells grown *in vitro* acquire unlimited proliferation potential through the bypass of the antiproliferative barrier of senescence. It is accepted that bypassing cellular senescence through the distinct alterations of pathways involved in its activation allows human somatic cells to undergo immortalization and acquire a growth advantage (44–47). As envisioned, one of the rate-limiting steps en route to full immortalization is the activation (or de-repression) of telomerase. Spontaneous telomerase re-activation in human somatic cells grown *in vitro* is a very rare-event, with a frequency of re-activation that varies across different human cell types (from $10^{-8/-10}$ in humans to 10^{-5} in mice). It is therefore accepted that telomerase re-activation is required to achieve complete immortalization since it is necessary to maintain telomere length and prevent replicative senescence. The

vast majority of cancer cells (~90%) have up-regulated telomerase activity, while the rest utilizes the alternative-telomere lengthening pathway (a homologous recombination-based lengthening) as a mechanism for telomere length maintenance. Furthermore, virtually all human cancers lack functional p53/pRb pathways, which are widely regarded as two of the key senescence signaling routes (48,49). These pathways often carry mutations in sets of genes that are known to collaborate *in vitro* to bypass the senescence response. In recent years, many groups have documented the presence of senescent cells induced by oncogenic signaling in several precancerous tissues obtained from humans and mice (23,50–53). These studies indicate that OIS is an authentic process that occurs *in vivo*. More importantly, these studies suggest that OIS is an active process that occurs in response to oncogenic stimuli and offers a protective mechanism against tumor development. Therefore, cellular senescence is viewed as a key early barrier in carcinogenesis (4).

In this context it is essential to clarify major differences between early passage human and rodent cells with respect to the senescence barriers that need to be bypassed to achieve full immortalization. Cells from small rodents (mice, rats and hamster) have a single barrier to immortalization, that can be readily bypassed via pRB pathway (mutational or epigenetic) or p53 (mutational) pathway inactivation. Human cells (fibroblasts and variety epithelial cells) require, in addition, bypass of telomere-driven replicative senescence through reactivation of telomerase (transcriptional derepression of hTERT) an extremely rare event. The differences originate from the fact that rodent cells have telomerase permanently 'on' even when irreversibly senescent (54).

Effector pathways

Cellular senescence pathways are believed to have multiple layers of regulation, with additional redundancy inherent in these layers (55–57). In addition to canonical signal transduction layers, regulation by miRNAs and methylation have recently been uncovered (15,58). Many of the functional studies in which a putative senescence gene is overexpressed indicate that a single gene/pathway is required for repair and subsequent reversion to senescence, suggesting that senescence is essentially a recessive phenomenon. Over all, most tumors have elongated telomeres via the up-regulation of telomerase activity and carry one or more inactivating mutations in the effector pathways. These mutations confer 'immortality' to tumors. If this property can be achieved by genetically altering proteins involved in senescence, environmental carcinogens may promote a similar phenotype and therefore should be carefully examined. The reasoning, however, is complicated; for example acute alterations of the senescence pathways, such as the inhibition of a tumor suppressor (pRb, p16INK4a), are often recognized by the cell as an unwanted proliferative signal and preventive senescence is triggered in response. It seems that chronic and/or sustained downregulation of a percentage of this signaling activity is more probably to achieve the goal of immortalization, but very little research has been conducted with regards to this topic. Furthermore, this mechanism is an essential portion of the hypothesis that environmental carcinogens may extend the lifespan of cells.

The dynamics of senescence exhibit two different steps: cell cycle arrest and further acquisition of senescence features, which include permanent arrest.

Senescence effector pathways converge at the point of cell cycle arrest through CDK inhibition. Therefore, most pathways known to be involved in senescent arrest impinge either directly or indirectly on this process. Namely, the most known effector pathways are the p16INK4a/pRB pathway, the p19ARF/p53/p21CIP1 pathway and the PI3K/mTOR/FoxO pathway (39,48,59–61), all of which exhibit a high degree of interconnection (Figure 1). Two pathways have been proposed to be responsible for the acquisition of irreversible arrest and senescence: the pRB pathway and the mTOR pathway. There is a high degree of redundancy among all of these effector pathways (Figure 1). If senescence program is not activated, cells are only transiently arrested with the possibility of resuming growth once the proliferation constraints have been eliminated (9,62). It has also been shown that if mTOR is activated under conditions of proliferative arrest, then arrest becomes permanent and the cell undergoes senescence (63,64). This can also be accomplished by producing permanent changes in the chromatin, especially at E2F transcription sites, which

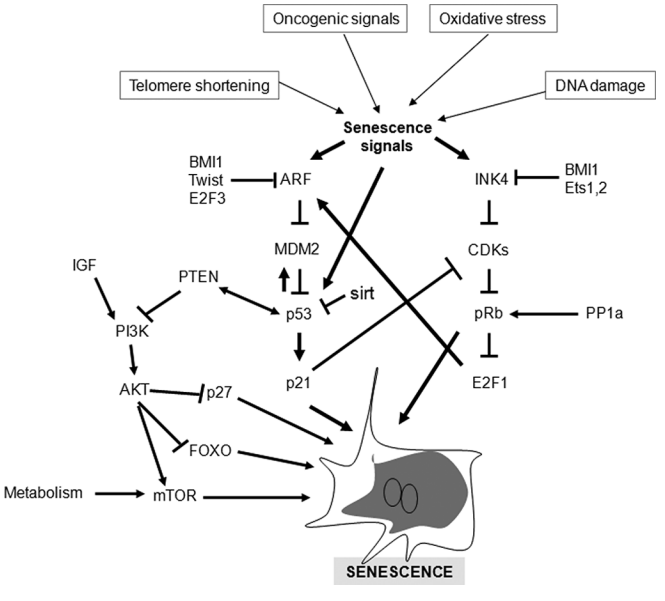

Figure 1. Simplified scheme of the effector pathways contributing to cellular senescence.

result in a blockade of transcription of proliferative genes (13). It has been shown that permanent inactivation of pRb, perhaps in combination with phosphatases (65), may signal for the differential recruitment of silencers to the heterochromatin of promoter sites. Human cells show heterochromatin compaction during senescence (SAHF), which is dependent on the pRb pathway (66). These SAHFs cause stable silencing of cell cycle genes and seem to be a factor in the stability of permanent arrest during senescence.

The p53 pathway

Replicative senescence, cellular stress or oncogenic Ras can activate p53 and promote cellular senescence, which limits the transformation potential of excessive signaling events (67–69). Inhibiting the function of p53 substantially extends the lifespan of several cell types in culture (70). Consistent with these findings, senescence is associated with the transactivation of p53 in cell cultures (71). Telomere shortening activates a DNA-damage checkpoint associated with genomic instability and leads to p53 activation *in vitro* and *in vivo* (72). Deletion of p53 attenuates the cellular and organismal effects of telomere dysfunction, which establish a key role for p53 as the gatekeeper of telomere shortening (72).

As expected, other p53 regulatory proteins are involved in senescence. Overexpression of MDM2 targets p53 for degradation and induces functional p53 depletion (73). Expression of p14ARF (INK4 alternative reading frame), another factor that is up-regulated during senescence and shares the INK4A locus with p16INK4a, releases p53 from MDM2 inhibition and causes growth arrest in young fibroblasts (73,74). ARF-defective mouse cells are efficiently immortalized (74,75), as do cells overexpressing MDM2.

Activation of p53 induces the up-regulation of the CDK inhibitor p21CIP1, which directly inhibits the cell-cycle machinery (49) and correlates well with the declining growth rates observed in senescent cultures. In human cells, depletion of p21CIP1 is sufficient to bypass senescence (76). However, in mouse embryo fibroblasts, the absence of p21CIP1 does not overcome senescence (77,78). This finding suggests that at least one additional downstream effector is needed for p53-induced growth arrest during senescence. Other p53 effectors, such as 14-3-3-sigma and GADD45 (both of which inhibit the G_2/M transition), or the downregulation of myc (79) are also potentially involved, thus underlining the redundancy of senescence effectors. It was also demonstrated that Ras modifies p53-dependent transcriptional activation in a quantitative, rather than qualitative manner and that the senescence response depends on factors other than p53 activation (9). p53 activation appears to be necessary for growth arrest but due to the possible requirement for additional signals is not sufficient to induce senescence.

The retinoblastoma pathway

The activities of tumor suppressors are mainly attributed to their ability to bind and inactivate the E2F family of transcription factors, which transactivates several genes encoding cell cycle proteins and DNA replication factors that are required for cell growth (80,81). pRb and its related proteins p107 and p130 are members of the pocket protein family (82). The pocket proteins are substrates for cyclin/CDK complexes (83) which in turn are inhibited by CDK inhibitors of the CIP/KIP and INK4 families of proteins. Both classes of inhibitors are up-regulated during cellular senescence (23), reducing pRb phosphorylation and thus preventing E2F inactivation.

Overexpression of pRb and some of the regulators of the pRb pathway, such as the CDK inhibitors, trigger a growth arrest which mimics the senescent phenotype (24). Moreover, inactivation of pRb by viral oncoproteins, such as E7, SV40 large T antigen and E1A, extends the cellular lifespan (84–86). Other members of the pocket protein family may also be involved. In mouse embryo fibroblasts (MEFs), p130 levels decrease as population doublings increase, and MEFs from triple pRb, p130 and p107 knockout mice are immortal (87). Nevertheless, a certain degree of complementation has been observed among the pocket protein family members (87); thus, it is difficult to assess the role of each protein in replicative senescence.

It is likely that pRb possesses more tumor suppressive activity than the other pocket proteins because mutations that alter p107 and p130 are very rarely observed in human cancers (88). Indeed, pRb seems to have a non-redundant role in tumor suppression and is thought to permanently repress E2F target genes during cellular senescence but not during quiescence. These observations suggest that loss of pRb, but not p107 or p130, results in a defective senescence response (89).

Given that CDK inhibitors of the INK4 (p16INK4a, p15INK4b, p18INK4c and p19INK4d) and CIP/KIP (p21CIP1, p27KIP1 and p57KIP2) families block the CDK inactivation of pRb (90), a loss-of-function of INK4 proteins would conceivably have similar consequences as a loss-of-function of pRb. Several types of human cells accumulate p16INK4a and/or p15INK4b protein as they approach senescence (91,92). Senescent fibroblasts potentially contain p16INK4a levels greater than early passage cells. The deletion or promoter methylation of p16INK4a is common in immortalized tumor cell lines (93), and several non-tumorigenic *in vitro* immortalized cell lines also lack functional p16INK4a protein. Expression of p16INK4a-specific antisense RNA in naive MEFs increases the probability that these cells will undergo immortalization (75). In accordance with this observation, mouse cells that are rendered nullizygous for p16INK4a via targeted deletion undergo immortalization more readily than normal control cells (94,95). However, these cells still exhibit normal senescence kinetics. p16INK4a knockout mice develop normally through adulthood and are fertile, which indicates that the individual INK4 proteins are not essential for development. However, p16INK4a deficiency results in a low susceptibility to spontaneous tumor development and increased tumor susceptibility under specific carcinogenic protocols (94,95). This may be due to the fact that mouse cells rely on ARF rather than p16INK4a for cellular senescence. Interestingly, Syrian hamster cells appear to be more similar to human cells by using p16INK4a instead of ARF as their primary senescence effector (96). It is possible that systems based on these cells to screen for senescence-bypassing carcinogens are more predictive of the human response than other rodent cells.

The polycomb group of proteins is critical for the transcriptional repression of the *INK4a-ARF* locus (Figure 1). Mouse embryonic fibroblasts deficient for the polycomb group protein BMI1 undergo premature cellular senescence due to the derepression of both the *INK4a* and *ARF* genes (97). The polycomb group proteins are chromatin remodelers that repress gene expression by shaping chromatin structure (98,99).

The Id family of helix–loop–helix transcriptional regulatory proteins coordinates cell growth and differentiation pathways; it also regulates G_1-S cell-cycle transitions. Although depending on the cell line, loss of Id1 increases the expression of the tumor suppressor p16INK4a but not ARF. Id1 depletion also reduces CDK2 and CDK4 kinase activity, which leads to premature senescence (100,101). Id1 directly inhibits p16Ink4a promoter activity via its helix–loop–helix domain but does not affect ARF. Therefore, Id1 may be a context-dependent inhibitor of cellular senescence via the repression of p16INK4a.

In line with this, Ras-induced activation of PPP1CA, the catalytic subunit of PP1α, is necessary to induce Ras-dependent senescence (102). PPP1CA stabilizes the active unphosphorylated form of pRb in a p53-independent manner. Unphosphorylated pRb will bind and inactivate E2F factors. This action blocks cell cycle progression and alters local chromatin (13) structure, resulting in the production of SAHFs. These transitions result in the accumulation of heterochromatin around E2F-responsive promoters in senescent cells, which stably silences E2F-regulated genes and forms SAHFs (13).

PI3K/AKT/mTOR/FoxO constitutes an important pathway regulating the signaling cascades of multiple essential biological processes (103–105). Many components of this pathway are genetically altered in cancer cells. AKT is a master kinase that phosphorylates MDM2 (among other proteins) and promotes its translocation to the nucleus, where it negatively regulates p53 function (106). One of the most conserved functions of AKT is its role in cell mass increase through the activation of the mTOR complex 1 (mTORC1 or the mTOR/raptor complex), which is regulated by both nutrients and growth factor signaling. mTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control (107). PI3K has been related to the induction of cellular senescence in several ways that are still not fully understood. Early works from Collado *et al.* (108), suggest that PI3K inhibition induces senescence through the activation of p27kip1. However, further works also indicated that the overexpression of active P110a (catalytic subunit of PI3K) or AKT induces OIS in primary cells in culture and *in vivo* (77,109–112). On the other hand, loss of PTEN triggers cellular senescence through a p53-dependent mechanism (51) and results in indolent prostate cancer. Therefore, concomitant or sequential loss of PTEN and p53 results in a dramatic acceleration of prostate tumorigenesis. Studies in murine mouse models have shown that p53 is the preferred mutation upon PTEN loss. In constitutively active AKT or PI3K transgenic models, an increase in benign lesions are observed if senescence is induced upon AKT activation (53,113).

AKT activation can also stimulate proliferation through multiple downstream targets and impinge on cell-cycle regulation. AKT phosphorylates some members of the FoxO family while they are present in the nucleus, thus creating binding sites for 14-3-3-sigma proteins that trigger their export from the nucleus. Through this mechanism, AKT blocks the FoxO-mediated transcription of target genes that promote apoptosis, cell-cycle arrest, and metabolic processes (Figure 1) (114,115).

FoxO transcription factors are an evolutionary conserved subfamily that regulates a number of cellular processes involved in cell-fate decisions in a cell-type- and environment-specific manner, including metabolism, differentiation, apoptosis and proliferation (116). A key mechanism by which FoxO determines cell fate is through regulation of the cell cycle machinery. FoxO plays a crucial role in regulating cellular senescence by controlling the expression of a number of cell cycle regulators, including p27kip1 (108). Moreover, overexpression of FoxO or p27KIP1 in primary mouse embryo fibroblasts can recapitulate this phenotype, promoting premature cell cycle arrest, changes in cell morphology and increases in senescence-associated markers. The ability of FoxO to induce G_0/G_1 arrest is lessened in p27Kip1 and p130 double deficient fibroblasts (117), suggesting that both p27Kip1 and p130 are important for mediating FoxO-dependent cellular senescence associated G_0/G_1 arrest. Further evidence of a role for FoxO in cellular senescence is supported by a recent *in vivo* study demonstrating that OIS also involves the repression of the PI3K–PKB signaling pathway and the induction of FoxO (118).

mTOR is an essential convergence point for the PI3K/AKT/FoxO pathways (119). mTOR is the master regulator of protein synthesis (120). It has been proposed that for growth arrest to become permanent (i.e. undergo senescence), a high level of mTOR activation is necessary (121,122). In fact, rapamycin treatment, which inhibits mTOR, can divert senescence into quiescence, allowing the cell to resume growth once conditions are more favorable (123,124). It has been proposed that this contribution is due to the function of mTOR as a sensor of cellular nutrients and energy status as well as growth factor signals. mTOR then integrates those signals and 'decides' whether the amount of metabolites and energy are sufficient to permit protein synthesis (107,125).

Carcinogen-induced bypass of cellular senescence

With these studies in mind, we identified a number of targets for which their alteration will contribute to immortalization. However, only a handful of genes are commonly measured: p53, hTERT and the INK4a/b locus. The rest of the genes are not commonly tested, and we do not know whether these genes are implicated in carcinogen-induced immortalization or to what extent they may contribute. Furthermore, to date, most carcinogen studies consider the initiation or progression of tumors as the measurable endpoint; however, they do not generally consider immortalization to be one of these endpoints. Immortal cells do not form tumors and need a further signal (oncogenic activation for example) to initiate carcinogenesis (Figure 2). Therefore, the identification of carcinogens is biased toward those chemicals that are able to produce alterations in several hallmark analyses and those capable of inducing a full-grown tumor. Therefore, we can expect that carcinogens altering a broad range of targets be more effective in these settings. Thus, DNA-damage (genotoxic) or methylating/demethylating agents (non-genotoxic) are easily identified since produce general changes in the genome. However, these searches come with a drawback, DNA-damage chemical compounds have been shown to induce senescence in a cell population, with only a few immortal (tumoral perhaps) clones arising from the whole culture. These clones are immortal due to DNA mutations (or epigenetic silencing) randomly occurring at a immortalizing gene site (1,2,96). The frequency of these immortal/tumoral clones is still high in comparison with spontaneous occurring immortal clones. However, it is expected that the same carcinogen hitting a naive culture that is already immortal (non-tumoral), either because stem properties of the targeted cell or because other non-carcinogenic compound is inhibiting only senescence (Figure 2), will induce a much higher level of tumoral clones from the cellular population, and would therefore behave as a much more potent carcinogen. This may also hold true for other compounds considered carcinogens but non-hazardous due to the low doses found in the environment, which may be reconsidered, since low doses of this compounds in the presence of a compound inhibitor of senescence might induce high tumorigenicity (Figure 2).

However, the literature regarding immortalization-only agents is very limited.

Genotoxic and non-genotoxic carcinogens

Chemicals are classified based on their carcinogenic capacity, and the IARC has categorized the carcinogenicity of all known chemicals (or agents) into four groups (http://monographs.iarc.

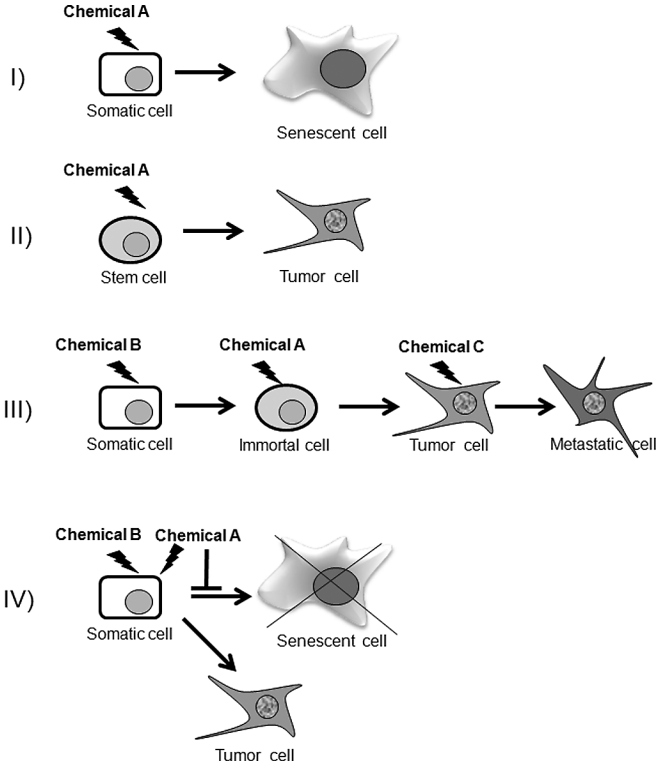

Figure 2. Proposed carcinogenic roles for different chemical compounds underlining the relevance of 'immortalization' compounds. (I) Compound acting on somatic cells at different levels (DNA-damage for example) Compound A may induce senescence. (II) The same compound A acting on a stem cell that does not have replicative constraints will induce tumorigenesis. (III) If the same compound A acts after an 'immortalization' compound (compound B) that overrides the senescence barrier, the compound will be carcinogenic in these somatic cells. (IV) Similar to III, but in this case, both compounds act in a mixture.

fr/ENG/Classification/index.php) ranging from carcinogenic in humans to most likely not carcinogenic in humans. Each carcinogen can be further classified based on its mode of action into the genotoxic carcinogen (GC) group or the non-genotoxic carcinogen (NGC) group. GCs are defined as chemicals or agents that directly initiate carcinogenesis via a direct interaction with DNA, thus initiating DNA damage and chromosomal aberrations that can be detected by genotoxicity testing. In contrast, NGCs are agents capable of inducing cancer via a secondary mechanism, such as the result of indirect action on DNA with the capacity to alter signal transduction pathways or gene expression. GCs can be detected using genotoxicity testing (Table 1), which detects changes to the cell at the molecular and cellular levels. These changes include mutations in genes, DNA strand breaks, formation of DNA adducts, chromosomal aberrations and aneuploidy, all of which can be detected using the validated methods listed in Table 1. Nowadays, sequencing of entire genomes through next generation sequencing technologies allows the identification of mutations generated by these GCs and the identification of specific altered pathways. The mechanism of GCs in the immortalization process is thought to be through direct inactivation (via point mutations and deletions) of the effector pathways. For example, the powerful mutagenic carcinogens *N*-methyl-*N*-nitrosourea (MNU) (CAS# 684-93-5) and benzo(a)pyrene (BaP) (CAS# 50-32-8) have been shown to be efficient immortalizing agents in a Syrian hamster dermal (SHD) cell transformation assay (129) through the direct inactivation of the tumor suppressors p53 and p16 (96). An inactivating p53 mutation was observed in 70% of the clones induced with BaP or MNU carcinogens in immortalized SHD cells. Most mutations were within the DNA binding domain of p53 in known as 'hot spot' codons that confer either inactivation or gain-of-function mutations (96,130). BaP has also been shown to immortalize human mammary epithelial cells (131), but the mechanism of complete immortalization is not known. A screen of p53 mutations in the two BaP-treated immortal mammary epithelial clones derived from the primary cell line identified no p53 mutations in exons 4–9, and high levels of p53 protein were observed via immunohistochemistry (132). This indicates that there may be other pathways or proteins involved in the immortalization processes.

Physical carcinogens (such as ionizing radiation) are also powerful immortalization agents with different mechanisms and frequencies in rodent and human cells. For example, x-rays, neutrons and gamma rays produce immortal clones in SHD cells, with a single dose in all immortal variants containing a CDKN2A/B locus deletion (96). In contrast, immortalization of human mammary cells by ionizing radiation is a relatively infrequent event (133). One such immortal variant generated by a fractionated cumulative dose (30 Gy in total) of IR (76-R30) showed a complete loss of p53 protein. Similarly, methyl sulphate, a powerful clastogen, is an efficient immortalizing carcinogen in mammalian SDH cells and Chinese hamster cells (129) and has a similar mode-of-action to that of ionizing radiation.

There is no evidence of a complete immortalization of primary human cells with genotoxic carcinogens, indicating that robust antiproliferative barriers exist in human cells. Such stringent barriers (OIS, replicative senescence and stasis as observed in HMECS) exist in human cells to act as tumor suppressors and to maintain genomic integrity. However, there is evidence of an increase in the tumorigenicity of spontaneously immortalized human oral keratinocytes infected with HPV-16/18 E6/E7 viruses when exposed to long-term BaP in culture

Table 1. List of known NGCs and their immortalization frequencies in mammalian cells

Carcinogen	Immortalization frequency	Mechanisms of immortalization
Nickel-derived compounds, including nickel chloride	9×10^{-7}	Epigenetic silencing of p16 (96)
Diethylstilbestrol	4×10^{-7}	Allelic loss and point mutation in ETRG-1 gene (126)
Reserpine	3×10^{-7}	Unknown but thought to be epigenetic (127)
Phenobarbital	2×10^{-7}	Reduces expression of the CDKN1A product p21 (128)

(134). The immortalization of human cells by the HPV-16 E6/E7 viruses occurs through the inactivation p53 or Rb1, respectively (135). However, the immortalized human oral keratinocytes are non-tumorigenic (134). It is the extended exposure to the genotoxic carcinogen BaP that increases the tumorigenicity and malignant phenotype of the immortalized human oral keratinocytes, possibly as a result of increased mutation rates and inefficient repair of DNA damage caused by the genotoxic carcinogens.

NGCs (Table 1) can induce immortalization in SHD cells at frequencies comparable with genotoxic carcinogens, probably through epigenetic mechanisms (96,136). Phenobarbital (PB) is a sedative that is also used as a hypnotic and antiepileptic agent. It is prescribed to people with epilepsy and has been classified as a class 2B carcinogen by IARC. PB was shown previously to promote cancer of the liver and thyroid in animal studies initiated by known carcinogens. It also promotes a reduction in the expression of p21CIP1 (the CDKN1A product), which was observed when PB was used in combination with N-nitrosodiethylamine. These results suggested a potential involvement of PB at the G_1-S cell cycle transition during liver carcinogenesis and senescence (128). Some data suggests that PB may also affect oncogenes, such as c-Myc, K-Ras and Fos, during rat liver cell line transformation (137). PB Induces cellular transformation in SHD cells at doses of 750 μg/ml, with a frequency of 2×10^{-7} (138), and induces morphological transformation in Syrian hamster embryo assays at a frequency of 1.11% at a dose range of 0.06–2.0 mM (139).

In contrast, nickel-chloride (a very potent NGC with a frequency of immortalization greater than that of BaP; 9×10^{-7} compared with 6×10^{-7}) induces the bypass of OIS by inactivating the p16INK4a-Rb pathway via the direct methylation of the p16INK4a tumor suppressor promoter and silencing the expression of the gene (96). Although it must be noted that the frequency of immortalization of nickel-induced HMECs is much lower compared with SHD cells. Other carcinogenic metals (such as arsenic, chromium and cadmium) are now thought to induce carcinogenesis in cells via epigenetic mechanisms (140); however, the exact mechanisms of the induction of complete immortalization in mammalian cells are not known (141). This is mainly due to the lack of accurate cell-based assays that are capable of measuring the carcinogenicity of NGCs (142). In addition, the current methods of classification have resulted in a high rate of false-positive data regarding NGCs present in IARC groups 1, 2A and 2B, which has initiated interest in devising better methods (cell-based and weight-of-evidence based) for the identification of NGCs. Essentially, the methods for the identification of chemicals capable of inducing only immortalization effects are not well developed.

Specific targeting of immortalization-related proteins

Telomerase

Telomerase activators readily promote the elongation of telomeres and extend the lifespan of the cell. There are data suggesting that acetaminophen activates telomerase (143–146), which could lead to the immortalization of cells. However, there is also data indicating that acetaminophen can inhibit CDK4 and CDK2, thus imposing a cell cycle checkpoint at G_1 and effectively blocking cellular proliferation. Another candidate could be bisphenol A, a chemical widely used in plastics (147–151). Like acetaminophen, it can activate telomerase and some data suggest that it induces cyclin A, cyclin D3, cdc2 and pRb. This activity is consistent with a bypass of senescence and an induction of proliferation (152). However, as mentioned previously, an acute induction of pRb phosphorylation might induce senescence by activating ARF and p53 (153). Chronic, low dose exposure has not been tested and the expected results are uncertain.

Several saponins from the plant genus *Astragalus*, including cycloastragenol (TAT2) and TA-65, have been used in traditional Chinese medicine and are currently sold as nutraceuticals with the promise of extending healthy life through the activation of telomerase. Cycloastragenol has been shown to transiently activate telomerase in CD8+ T lymphocytes from HIV-infected human donors, retarding telomere shortening and improving proliferation and the antiviral response (154). TA-65 has been shown to moderately activate telomerase in human keratinocytes, fibroblasts and immune cells in culture; furthermore, TA-65 diminished the percentage of senescent CD8 T lymphocytes *in vivo* in the absence of any adverse events observed in the human subjects (155). Studies with TA-65 in mice demonstrated that the compound increased the average telomere length, thus decreasing the percentage of critically short telomeres. Furthermore, the dietary supplementation of female mice with TA-65 led to an improvement in glucose tolerance, osteoporosis and skin fitness without increasing global cancer incidence (156). Although these plant saponins have been historically used in traditional Chinese medicine and are currently used as nutraceuticals, no detrimental effects were reported until recently. More research is necessary to ensure that these saponins are not increasing cancer risk through the activation of telomerase in combination with other chemicals.

Cotinine, a nicotine metabolite found in tobacco, exhibits a biological half-life 10 times longer than that of nicotine and has been shown to induce abnormal cell proliferation through the reactivation of telomerase in human vascular smooth muscle cells in a dose-dependent manner (157). Although there is some controversy about the effects of the isoflavone genistein, which is present in many *Fabaceae* beans, it has been shown to enhance telomerase activity at physiologically achievable concentrations (~1 μM) in prostate cancer cells through the activation of STAT3, Signal transducer and activator of transcription 3, (158). Nevertheless, at higher pharmacological concentrations (>10 μM), genistein has been shown to inhibit telomerase in all cell lines analysed (159). Thus, depending on the physiological concentration, the compound can have a bilateral effect on telomerase activity in cancer cells. *Ginkgo biloba* extracts are currently used as nutraceuticals in many food supplements and have been shown to induce telomerase activity, resulting in a reduction of endothelial progenitor-cell senescence in a dose-dependent manner. The mechanism through which the ginkgo

extract induces telomerase activity is not well understood, but the PI3K signaling pathway seems to participate (160).

Resveratrol, a stilbenoid that is produced by several plants and is currently used in food supplements and cosmetics, has been shown to activate telomerase in human mammary epithelial and endothelial progenitor cells, most probably through the up-regulation of SIRT1 or the activation of the AKT signaling pathway (161–163).

Although the most obvious effect of telomerase on tumor promotion is the facilitation of the bypass of the replicative senescence barrier that limits the number of divisions of the tumor cells through telomere stabilization, telomere attrition in the absence of telomerase activity can also favor the onset of a malignant phenotype. In fact, short telomeres can give rise to dicentric chromosomes, which can undergo several rounds of chromosomal bridge-breakage-fusion cycles during cell division, causing a high degree of chromosomal instability. In the presence of wild type p53, cells with highly rearranged genomes will enter crisis and, subsequently, die. Nevertheless, in the absence of p53 activity, alterations resulting from these bridge-breakage-fusion cycles would eventually increase the mutability of the genome, thereby accelerating the appearance of a malignant phenotype (164). The subsequent recovery of telomerase activity would eventually facilitate the reconstruction of longer telomeres and the fixation of the aberrant karyotypes that favor malignant phenotypes (165). Substantial evidence for this hypothesis is still lacking, but several circumstantial studies incorporating comparative analyses of premalignant and malignant lesions in the human breast point in this direction (166,167). This evidence highlights an essential point of this review: the combination of otherwise innocuous chemicals can give rise to pro-tumorigenic (or tumorigenic) phenotypes.

Thus, telomerase inhibitors may have a potential pro-carcinogenic role if individuals are exposed during the early phases of the process of tumorigenesis or under specific molecular or cellular circumstances. Many natural compounds have been identified as telomerase inhibitors, including allicin [an organosulfur compound found in garlic (168)], curcumin [a compound found in the spice turmeric (169)], silibinin [found in *Silybum marianum* (170)], sulforaphane [found in cruciferous vegetables, such as broccoli or cabbages (171)], EGCG [epigallocatechin gallate, found in tea (172)], helenalin [a lactone present in Arnica plants (173)], rubromycin [found in *Streptomyces collinus* (174)], among others. Nucleoside analogs used in HIV treatment, such as AZT, have also been shown to inhibit telomerase (175).

p53 is the gatekeeper of cellular stress. Its inhibition extends cellular lifespan and is necessary to bypass OIS. A number of chemical inhibitors, such as pifithrin, have been reported to directly bind and inhibit p53 activity (176). Although the exposure to pifithrin is limited because it is a laboratory product, it is expected that some chemicals either from nature or syntesized by man can produce the same effects.

The effect of antioxidants on p53 is clear (177–179). While superoxide dismutase (SOD) which converts $O_2^{\bullet -}$ to H_2O_2, was found to increase p53 activity, catalase, a scavenger of H_2O_2, inhibited p53 activation. Interestingly, aspirin, a scavenger of •OH, suppressed the activation of p53 (180). Increased formation of •OH enhanced p53 activation at the protein level but not at the transcriptional level (181). Maehle *et al.* (182) found that *p53* gene structure and expression was altered in human epithelial cells after exposure to nickel; however, in contrast, a low incidence of point mutations was detected in the *p53* tumor suppressor gene isolated from nickel-induced rat renal tumors. Regarding the effects of arsenic on p53, various studies have reported conflicting results spanning the range of arsenic demonstrating no effect on p53 to arsenic inducing p53 phosphorylation and, ultimately, leading to a decrease in p53 expression (183–186). Another mechanism by which metals affect p53 is via zinc substitution, which is essential for the binding of p53 to DNA. Metals substituting zinc can inactivate p53 without mutation or oxidation. Several studies have confirmed that mutations arise in p53 following exposure to NO• (187). Experiments have also indicated that exposure of cells to a high level of NO• and its derivatives during chronic inflammation in the absence of wild-type p53 and therefore negative iNOS regulation may increase susceptibility to cancer. There is an association between increased iNOS expression and G:C to A:T transition mutations in p53 in stomach, brain and breast cancers. NO• and its derivatives are therefore capable of causing mutations in cancer-related genes and therefore act as both an endogenous initiator and a promoter in human carcinogenesis (188,189).

Sodium-selenite increases p53 promoter methylation and also exhibits many additional global methylation effects (190,191). A reduction in the levels of p53 contributes to immortalization at the expense of compensatory effects in other genes.

Resveratrol increases the catalytic activity of Sirt1, promoting the deacetylation of p53 (192). Resveratrol is a phytochemical that partially prevents mitochondrial senescence induced in the lung by benzopyrene (193) but shows potential in preventing cancer and other diseases resulting from oxidative stress (194–197). Therefore, it seems plausible that the long-term benefits may outweigh the possible damage.

Although p53 is the central player in a network that senses cellular stress and generates an adequate response to the insult, other players exist both upstream and downstream of p53, the alterations of which may affect the final output of the network (Figure 1). For example, Ser20 phosphorylation is a key phosphor-acceptor site in the p53 transactivation domain that has been shown to be induced in an ATM-dependent manner upon exposure to X-rays, a CK1-dependent manner upon virus infection, and an AMPK-dependent manner upon perturbation of adenosine monophosphate/adenosine triphosphate ratios (198). Environmental compounds that inhibit these kinases could potentially inhibit the activation of p53 under stress conditions and facilitate the onset of the transformation process. Caffeine is an alkaloid present in many plants that inhibits the checkpoint kinases ATM (ataxia-telangiectasia mutated gene) and ATR (ataxia-telangiectasia and rad3-related gene) (199) and attenuates the activation of p53 via ser20 phosphorylation (200).

mTOR

Although maintaining its activity seems essential to 'finalize' the output of the senescent phenotype, acute mTOR inactivation has been used as antitumor therapy and promising results in a few specific tumor types (201–204). Therefore, although mTOR inhibitors (or mTOR activation by inhibiting PI3K or AKT), such as rapamycin, AKT inhibitors or PI3K inhibitors, could theoretically contribute to immortalization, this pro-tumorigenic effect can be counteracted by the effects of inhibiting an important proliferation pathway. However, the effect of a chronic, low dose exposure of mTOR inhibitors may be unexpected if cellular circumstances are appropriate to facilitate immortalization, particularly in cells that are not terminally arrested or in an increasing proportion of cells transitioning to senescence upon aging. Limited data have shown that lead can inhibit mTOR (205), gold nanoparticles can inhibit mTOR and Akt activation (206,207) and silver nanoparticles can inhibit Akt activation (208–211), making these chemicals possible candidates for

immortalization agent classification via the regulation of mTOR signaling. Lead is currently ubiquitous in the environment and gold and silver nanoparticles are probably already widespread in the environment or soon will be due to the large increase in their use over the past few years.

As with many other compounds (212), global methylation may alter genes involved in immortalization but these effects will probably be compensated for. For example, Genistein reduces the levels of p16 but also reduces hTERT mRNA expression through an increase in E2F1 (213). However, no chronic low dose exposure studies have been performed.

Oxidative stress and senescence

Since the proposal of the radical theory of aging by Harman (214), recent studies have suggested that the accumulation of ROS and oxidative damage are closely involved in senescence (5,215–219). ROS, such as the superoxide anion and hydroxyl radical, are produced during cellular metabolism, mainly in the mitochondria. ROS are also produced in response to different environmental stimuli, such as UV, IR, chemicals, hyperoxia or hydrogen peroxide treatment. Abnormal ROS accumulation and its effects on intracellular macromolecules (oxidation of lipid, protein and DNA) provoke cumulative damage at the cellular, tissue and organismal level. Mild oxidative stress (e.g. treatment with low concentrations of hydrogen peroxide) is enough to induce senescence in primary cells. Interestingly, premature senescence induced by culture-stress or oncogene-induced stress is associated with oxidative damage in cells (220).

Notably, increased ROS accumulation is also observed during replicative senescence. The replicative potential of both murine and human fibroblasts are significantly extended under low oxygen and are associated with less oxidative damage than that observed under normoxia (O_2 20%) (221). Immortalized cells suffer from less oxidative damage than primary fibroblasts when cultured at 20% O_2. Moreover, immortalized cells are more resistant to the deleterious effects of hydrogen peroxide than primary cells. Thus, the ability to resist oxidative stress could be a clue to explaining the immortality of cancer cells.

Several radical scavengers can protect cells against oxidative stress. The SOD enzyme converts superoxide anions into hydrogen peroxide, while hydrogen peroxide can be detoxified by catalase. Consequently, these antioxidant enzymes can impact both the proliferation of primary and immortal cells because they should counteract the effects of ROS. The ability of SOD to bypass senescence has been well studied and established in various cells and/or organisms. Increased expression of SOD can extend the life span of primary fibroblasts (222). Conversely, knockdown of SOD using siRNA induces premature senescence accompanied by p53 activation. Transgenic flies overexpressing SOD (223) or the detoxifying enzyme catalase (224) present with an extended organism life span. Although it is clearly established that these antioxidant scavengers are essential for the proliferation of immortal cells, to date, little is known about the specific chemicals affecting senescence via oxidative stress modulation.

First, N-acetyl-cysteine (NAC) is a well-known radical scavenger that also interferes in the ras signaling pathway. Ras-induced senescence in MEF was bypassed upon NAC treatment (225), whereas another group has shown that neoplastic transformation by Ras was perturbed upon NAC treatment (226). It is possible that NAC might be carcinogenic in a cellular context that remains to be clarified.

Coenzyme Q10 (coQ10) is another candidate. CoQ10 is an essential component of the mitochondrial respiration complex I. It is well known that the level of coQ10 in various tissues, especially in the heart, declines during organismal aging, including humans. This observation partially explains why some people favor the daily oral intake of coQ10 as a supplement. However, no clear scientific evidence has defined its effect on human longevity. There are several opposing reports on its effects on longevity in some model systems. While a coQ10 deficient diet significantly extends the life span of *Caenorhabditis elegans* (227), a lack of coQ10 shortened the longevity of *Drosophila* (228). It is noteworthy that mice under oral coQ10 treatment apparently displayed shorter survival rates than those maintaining a standard diet (7%). Histological analysis revealed that the major cause of death of these mice was an increased incidence of cancer, including hepatocellular carcinoma and malignant lymphoma.

Iron is an essential metal in mammals for the transport of oxygen by hemoglobin and for the function of many enzymes including catalase and cytochromes. However, the 'free' or 'catalytic' form of iron mediates the production of ROS via the Fenton reaction and induces oxidative stress. 'Free' iron is quite cytotoxic as well as mutagenic and carcinogenic. Ferric nitrilotriacetate induces oxidative damage in renal proximal tubules, which is a consequence of a Fenton-like reaction that ultimately leads to a high incidence of renal cell carcinoma in rats (229,230). It may be partially explained by a loss of heterozygosity in the INK4 locus with a modulated methylation status (231).

Finally, oncometabolites could affect oxidative damage and may be a hot topic for study. Kondoh *et al.* (232) previously reported that the glycolytic enzyme PGAM immortalized primary MEFs and reduced oxidative damage. It is reasonable to speculate that the modulation of PGAM activity would have a great impact on the process of tumorigenesis. Recent reports have suggested the detailed molecular mechanisms regarding how enhanced PGAM activity could attenuate oxidative damage. Thus, the ectopic expression of PGAM downregulated mitochondrial respiration activity by ~30% (233). Hitosugi *et al.* (234) reported that 3-phosphoglycerate, a substrate of PGAM, binds to and inhibits 6-phosphogluconate dehydrogenase in the oxidative pentose phosphate pathway. In contrast, 2-phosphoglycerate, a product of the PGAM reaction, activates 3-phosphoglycerate dehydrogenase to provide feedback control of 3-phosphoglycerate levels. Pentose phosphate pathway is essential for the generation of reduced nicotinamide adenine dinucleotide phosphate as an antioxidant. Moreover, another metabolite of glycolytic pathway, phosphoenolpyruvate, could bind to PGAM to increase its catalytic activity by over 100-fold. Thus, some glycolytic metabolites, such as 2-phosphoglycerate and PEP, may be candidate carcinogens because they act as a booster for glycolysis. Other metabolic enzymes have also been related to senescence. The mitochondrial gatekeeper pyruvate dehydrogenase is a critical mediator of B-Raf-induced senescence, which is also dependent of the induction of pyruvate dehydrogenase activating enzyme pyruvate dehydrogenase phosphatase (PDP2) and suppression of pyruvate dehydrogenase inhibitory kinase PDK1 (235). On the other hand therapy-iduced senescent cells seem to have enhanced Warburg effect, the non-oxidative breakdown of glucose, which seems to be related to pyruvate kinase 1 (236). Therefore, chemicals interfering with this signaling are also candidates to interfere senescence.

Many antioxidant molecules (vitamin C, flavonoids, carotenoids, selenium, etc.) are used as 'friends' against cancer: how it is possible that they can contribute to tumorigenesis? We do not know how it is possible that while the cells are not genetically modified, antioxidants may help to prevent the cells from entering into senescence and thus increasing the fitness of the

cell and the organism. However, with time, the age of the organism along with many cells that carry mutations under these conditions may allow antioxidants to open a back door to tumorigenesis. This is essentially a derivation of the hypothesis of pleiotropic antagonism. However, in any case, this hypothesis remains to be experimentally tested.

Inflammation may contribute to immortalization

In the 19th century, Virchow (237,238) postulated that cancer was linked to inflammation. Epidemiological studies have noted that chronic inflammation predisposes humans to different forms of cancer, and currently, this is an accepted paradigm (239,240). In the last few years, two different molecular pathways that link cancer and inflammation have been identified: the intrinsic and extrinsic pathways. In the extrinsic pathway, inflammatory conditions, such as infections, autoimmune diseases and those of unclear origin, induce chronic inflammation and increase cancer risk. In the intrinsic pathway, genetic events that cause neoplasia simultaneously initiate the expression of proinflammatory circuits. In both cases the key orchestrators of the inflammation and tumor progression are infiltrating leukocytes, transcription factors, cytokines and chemokines that share many factors with senescence-associated secretory phenotype (SASP). Moreover, especially in colon cancer, treatment with non-steroidal anti-inflammatory drugs protects against cancer development (241–243). In addition, NFkB pathway activation is a frequent event in carcinogenesis and a requirement for inflammation and tumor promotion (244). Inflammation can contribute to immortalization via two different ways: one, by abolishing the CDK inhibitor expression during senescence and allowing tumor progression, or two, by inducing the immunosurveillance of senescent cells.

Different groups have demonstrated that inflammation is necessary for tumorigenesis to occur in models where tumorigenesis is activated by different oncogenes. In the case of mouse models of pancreatic cancer, an inflammatory event is required at the same time as a KRas mutation is induced to allow the development of pancreatic ductal adenocarcinoma (245). Pancreatitis induced by caerulein contributes to tumor progression by abrogating the senescence barrier of low grade murine pancreatic intraepithelial neoplasia and the appearance of proliferating markers, such as Ki67, inversely correlate with the expression of senescence markers (SA-β-gal and p16INK4a). The authors show that OIS can be inhibited by limited episodes of pancreatitis but can reappear after the pancreatitis-induced damage has partially subsided (242). Moreover, the treatment of those mice with sulindac, a non-steroidal anti-inflammatory drug, dramatically reduced the number and size of high grade lesions, suggesting that inflammation is a key contributor to mPanIN promotion, formation and progression to murine pancreatic ductal adenocarcinoma. Similar results are shown in PanIN in human patients suffering from chronic pancreatitis that were treated with anti-inflammatory drugs. A second model of accelerated tumor formation with concomitant KRAs activation and a loss of p*Rb* tumorigenesis is associated with an induction of acute pancreatic inflammation. Again, coexpression of senescence (SA-β-gal and p16INK4a, p19ARF, IGGBP7, caveolin-1 and p15INK4b) and proliferative markers (ki67) suggests that OIS is bypassed, allowing the progression to high grade PanIN and PDAC (246).

In a model of prostate cancer, the overexpression of the *PIM1* oncogene alone or together with the loss of one *PTEN* allele induces senescence in high grade lesions with visible markers of senescence (p16, p21CIP1, p19ARF) only in the absence of inflammation. In contrast, upon hormone treatment, overexpression of *PIM1* increases inflammation, and the high grade mPIN1 lesions do not exhibit senescence markers (247).

Inflammation can also trigger the immunosurveillance of senescent cells. *In vivo* temporal restoration of endogenous p53 function in mouse tumor cells trigger their entry into senescence (with the expression of SA-β-gal, p15INK4b, p16INK4a and DcR2), followed by efficient clearance by the immune system (248). This implies that the temporal sequence of events between inflammation and senescence is essential for the output of the physiological process unchained and also suggests a novel mechanism of tumor suppression involving cooperative interactions between a tumor cell senescence program and the innate immune system.

How does inflammation influence senescence? The effect can be dependent on the cellular and molecular context. One of the mechanisms is thought to be through the biological effects of some cytokines, such as macrophage migration inhibitory factor (MIF). In cases of injury, surrounding inflammation releases many factors that will de-repress the arrest of somatic cells and allow local proliferation to close the wound. One of these cytokines is MIF, which is able to bypass p53-induced arrest by inhibiting p21CIP1 transcription and increases the ratio of immortalization in MEFs (249). If the effect is temporal, there is not much damage accumulation; however, in the case of chronic inflammation, MIF is present and a sustained downregulation of p53 increases the chances of tumorigenesis. Therefore, environmental chemicals that chronically maintain local inflammation can contribute to cancer by over-riding senescence.

The other way around; how senescence may contribute to tumorigenesis

It is almost always assumed that senescence is the opposite of immortalization and that to immortalize a cell it is necessary to bypass senescence. However, paracrine effects are induced by senescent cells toward their neighbors that can contribute to tumorigenesis, including the immortalization of cells that are genetically competent for senescence. This potential problem may be caused by certain cytokines that are known to be released by senescent cells.

Recent evidence in fibroblast and epithelial cells has shown that cellular senescence is accompanied by an increase in the secretion of multiple factors that participate in cell signaling (250). This phenotype has been designated the 'SASP' (215,251). Among these factors are interleukins (IL-1, L-1β, IL-6, IL-7, IL-8, IL-11, IL-13 and IL-15), metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-10, MMP-12 and MMP14), monocyte chemotactic proteins (MCP-1, MCP-2 and MIP-1α), insulin growth factor binding proteins, VEGF, angiotensin, oncostatin, among others. Thus, senescent cells can alter their microenvironment for as long as they persist. The SASP has beneficial and deleterious effects if left unchecked because cytokines are mainly pro-inflammatory molecules (252). As stated previously, inflammation is a good response by the immune system when an emergency situation appears and needs an urgent solution. The problem arises when inflammation persists as a chronic process. It has been shown that senescent cells promote the proliferation of premalignant epithelial cells *in vitro* and *in vivo* (253,254).

Multiple SASP components have been identified that mediate paracrine senescence, including TGB-β family ligands, VEGF, CCL2 and CCL20 (255). On the other hand, as senescent cells may accumulate according to the age of the individual, a low basal level of senescent cells might be constantly present in every

organism. As the organism loses the ability to provide an efficient immune response, senescence then becomes a handicap. Among SASP regulators are DNA-damage response proteins, p38MAPK activation, IL-1α and microRNAs that act epigenetically (i.e. miR-146a/b). For example miR-146a/b plays a key role in modulating the innate immune response, which involves the NKkB pathway (256), and the increased expression of miR-146a in endothelial cells that occurs during replicative senescence (257).

Furthermore, compounds inducing cellular senescence could contribute to tumorigenesis in other neighboring cells depending on the cellular context. However, the extent of this hypothesis needs to be proven experimentally.

Many chemical agents or types of radiation can induce cellular senescence (258). Oxidative agents are among the more potent senescence inducers. Moderate doses of doxorubicin induced a senescent phenotype in 11 out of 14 tumor cell lines that were analysed independent of p53 status (259). A similar effect has been observed in lines from human tumors treated with cisplatin (260), hydroxyurea (261) and bromodeoxyuridine (262,263). Under equitoxic doses, the strongest induction of a senescent phenotype was observed with DNA-interacting agents and the weakest effects were observed with microtubule-targeting drugs. A medium response was observed with ionizing radiation. Induction of senescence by the chemicals was dose dependent and correlated with growth arrest observed in the cultures (258,261–263). The compound-induced senescent phenotype in tumor cells was not associated with telomere shortening and was not prevented by the expression of telomerase (264).

Cross-talk between replicative immortality and the other hallmarks of cancer

Given that the carcinogenicity of low dose exposures to chemical mixtures in any given tissue will probably depend upon simultaneous instigation of several important tumor promotion mechanisms and the disruption of several important defense mechanisms, it was felt that a better way of visualizing the potential synergies of combinations of chemicals will ultimately involve a thorough review of disruptive actions across the full range of mechanisms that are known to be relevant in cancer biology. Accordingly, we undertook a thorough cross validation activity to illustrate the importance of the prioritized target sites for disruption that this team has identified (i.e. across multiple aspects of cancer's biology) and to illustrate the extent to which the prototypical chemical disruptors that we identified (i.e. also disruptive to other mechanisms that are also relevant to carcinogenesis).

There is a strict relationship between pathways and chemical agents involved in the acquisition of replicative immortality and the achievement of the other cellular capabilities that, according to Hanahan *et al.* (165), distinguish neoplastic cells. Telomerase is a multifaceted complex that plays a role in several biological processes (265). A large body of evidence indicates that the induction of hTERT expression not only leads to telomerase activation, and thus telomere maintenance and replicative immortality, but also has a positive role in the achievement of cellular capabilities as different as angiogenesis or immune system evasion (Table 2), hence promoting tumorigenesis through many different routes (266). Similarly, p53 and pRB are essential for a proper cellular functionality and their inactivation causes the acquisition of a wide spectrum of cancer-related features (Table 2). A more complex relationship is present between mTOR inactivation and the acquisition of other cancer hallmarks. In fact, if mTOR inactivation is viewed as a possible therapeutic strategy contrasting

Table 2. Involvement of the cellular pathways promoting replicative immortality in the development of the hallmarks of cancer identified by Hanahan *et al.* (165)

Replicative immortality priority targets	Deregulated metabolism	Evasion of antigrowth signaling	Angiogenesis	Genetic instability	Resistance to cell death	Immune system evasion	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor microenvironment
Telomerase activation	+ (266)	+ (266)	+ (266)	+ (266)	+ (267)	+ (266)	+ (266)	+ (266)	+ (266))	– (268)
P53 inactivation	+ (269)	+ (270)	+ (271)	+ (272)	+ (273)	+ (274)	+ (275)	+ (276)	+ (277)	+ (278)
pRb inactivation	+ (279)	+ (280)	+ (281)	+ (282)	+ (283)	0	+ (284)	+ (285)	+ (286)	+ (287)
mTOR inactivation	+ (288)	+ (289)	– (290)	+ (291)	– (292)	– (293)	– (288)	– (294	– (295)	+/– (278)

Targets that were found to have opposing actions in a particular hallmark (i.e. anticarcinogenic) were denoted using '–', while targets that were found to have promoting actions in a particular hallmark (i.e. pro-carcinogenic) were denoted using '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both pro-carcinogenic potential and anticarcinogenic potential), the symbols '+/–' were used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, were denoted using '0'.

Table 3. Involvement of disruptors promoting replicative immortality in the development of the hallmarks of cancer identified by Hanahan *et al.* (165)

Replicative immortality prototypical disruptors	Deregulated metabolism	Evasion of antigrowth signaling	Angiogenesis	Genetic instability	Resistance to cell death	Immune system evasion	Sustained proliferative signaling	Tissue invasion and metastasis	Tumor promoting inflammation	Tumor microenvironment
Acetaminophen	0	0	- (301)	- (301)	– (302)	0	+/– (303,304)	– (303,304)	+/– (305,306)	0
Cotinine	0	0	+ (307)	+ (302)	+ (308)	0	+ (308)	0	– (309)	0
Nitric oxide	0	+ (310)	+ (297)	+ (311)	+/– (312)	– (313)	– (314)	+/– (315)	+ (316)	+ (298)
Na-selenite	0	0	– (317)	- (318)	+/– (319,320)	0	+/–	– (321)	– (322)	0
Nickel chloride	+ (323)	+ (324)	+ (325)	+ (326)	+/– (327,328)	0	+/– (329,330)	0	+ (331)	+ (332)
Lead	0	0	0	+ (333)	– (334)	0	+ (335)	0	+ (336)	0

Disruptors that were found to have opposing actions in a particular hallmark (i.e. anticarcinogenic) were denoted using '–', while targets that were found to have promoting actions in a particular hallmark (i.e. pro-carcinogenic) were denoted using '+'. In instances where reports on relevant actions in other hallmarks were mixed (i.e. reports showing both pro-carcinogenic potential and anticarcinogenic potential), the symbols '+/–' were used. Finally, in instances where no literature support was found to document the relevance of a target in a particular aspect of cancer's biology, were denoted using '0'.

several cellular processes sustaining tumorigenesis (Table 2), evidence has been reported that it can play a role in promoting metabolism alterations, evasion of antigrowth signaling and genetic instability, which favor neoplastic transformation (261–263). Moreover, mTOR can have both a pro- or anti-tumor activity regulating autophagy in cancer and tumor stroma cells (278).

Among the compounds promoting replicative immortality, nitric oxide, a physiological cellular metabolite, can mediate tumor formation by stimulating angiogenesis through the modulation of VEGF (296,297), sensing the inflammatory mediators present in tumor microenvironment (298). Moreover, nitric oxide can influence the cell's decision to survive or die in opposite ways, depending on the cellular context (299) and, similarly, it can have conflicting effects on metastasis formation (300). Cotinine, a nicotine metabolite found in tobacco, promotes tumorigenesis mediating the acquisition of different cancer hallmarks (Table 3); however, it seems to be effective in preventing inflammation (337). The environmental compounds lead and nickel have a potent effect on the different processes leading to cancer development; both are capable to generate oxidative stress and damage DNA leading to genetic instability (326,333) and tumor promoting inflammation (326,337). The reported stimulatory activity of the widely used drug acetaminophen (also known as paracetamol) toward telomerase (143) stimulated the investigation of its possible pro-tumoral effect; however, this compound was found to exert overall a protective effect on tumors (Table 3). Analogously, the inorganic Na selenite is generally regarded as a protective agent (338), despite its stimulation of cellular proliferation (339).

Conclusions

Senescence is a mechanism imposed to limit the number of divisions that somatic cells can perform thus becoming permanently arrested. The mechanism possesses a high degree of redundancy. Furthermore, attempts to induce the system to bypass senescence are usually recognized as unwanted signals and trigger a senescence response. However, these conclusions are based on the interpretations of experimental designs in which acute molecular or cellular alterations are produced. There are very few, if any, experiments regarding the effects of chronic, low dose alterations. There are even less studies considering the different cellular and molecular contexts that can arise over the course of a lifetime. It is necessary to design cellular and organism model systems that allow for this type of test to explore the effects of environmental chemical carcinogens at low doses in mixtures.

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