REVIEW

The p16^{INK4a}-RB pathway : molecular link between cellular senescence and tumor suppression

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Abstract : The p16^{INK4a} tumor suppressor protein functions as an inhibitor of CDK4 and CDK6, the D-type cyclin-dependent kinases that initiate the phosphorylation of the retinoblastoma tumor suppressor protein, RB. Thus, p16^{INK4a} has the capacity to arrest cells in the G1-phase of the cell cycle and its probable physiological role is in the implementation of irreversible growth arrest termed cellular senescence. Cellular senescence is a state of permanent growth arrest that can be induced by a variety of stresses such as DNA-damage and aberrant mitogenic signaling in human primary cells. In contrast to normal cells, the function of the p16^{INK4a} gene or its downstream mediators is frequently deregulated in many types of human cancers, illustrating the importance of cellular senescence in tumor suppression. Here we discuss the molecular mechanisms that direct cellular senescence and reveal its potential for tumor suppression.
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INTRODUCTION

Cell division is a fundamental process whose error-free execution is essential for the continuity of all living organisms. In higher eukaryotes, there is an additional need for cell to stop dividing in order to differentiate and to maintain the correct balance of tissues and cell types (1). Aberrant cell growth therefore underlies many hypo-and hyper-proliferative disorders, including cancer, and a better understanding of the mechanisms involved could lead to new strategies for treatment and prevention of cancer (2).

In contrast to cancer cells, most human normal somatic cells permanently stop dividing after a finite number of cell divisions in culture and enter a state termed cellular or replicative senescence (3). These cells are irreversibly arrested in the G1 phase of the cell cycle and no longer able to divide despite remaining viable and metabolically active for long periods of time (Figure 1). Most tumors contain cells that appear to have bypassed this limit and evaded replicative senescence. Immortality, or even an extended replicative lifespan, greatly increases susceptibility to malignant progression because it permits the extensive cell divisions needed to acquire successive mutations. Thus, cellular senescence may act as a barrier to cancer and play an important role in tumor suppression (4).

A number of hypotheses have been proposed to explain the mechanisms of cellular senescence, and they can be grouped into two broad categories (5). One set proposes that the loss of proliferative potential is due to random accumulation of damage or stress, while the other proposes that genetically programmed processes. Although numbers of reports suggest that that senescence in human cells is genetically controlled , at least, through a cell division counting mechanism (telomere-shortening)(6), accumulating evidences strongly suggest that it can also be induced by variety of extrinsic physiological stresses (7-9). Thus, the truth

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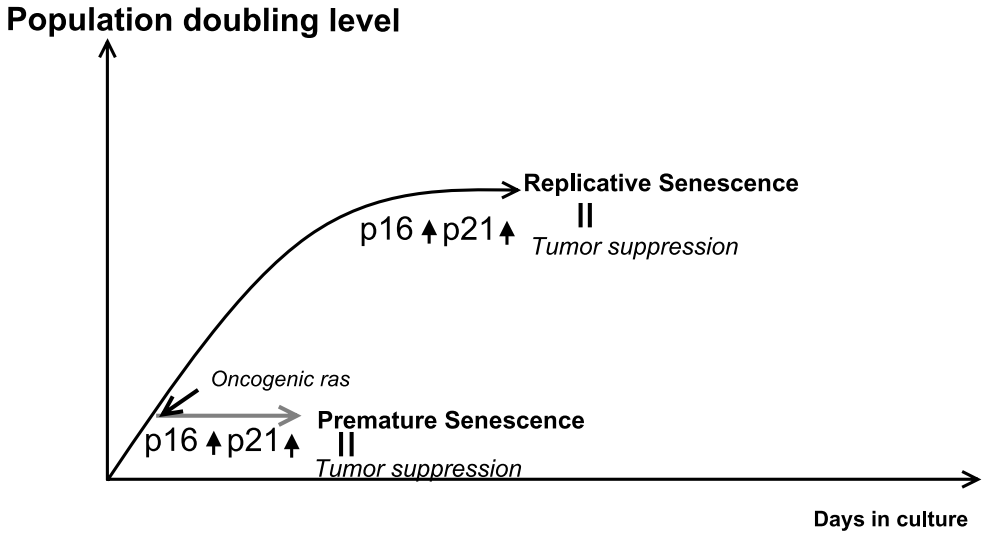

Figure 1. Tumor suppression by cellular senescence
Expression of CDK inhibitors, p 16^{INK4a} and p 21^{Cip1}, is induced in both replicative senescence and premature senescence.

probably lies somewhere in between. Indeed, it has become apparent that the $p16^{INK4a}$-RB tumor suppressor pathway is activated by a variety of distinct physiological stresses, and is playing a central role in induction and maintenance of cellular senescence.

In this review, we will discuss possible mechanisms that induce cellular senescence and elucidate how these mechanisms are perturbed in cancer cells. We also discuss the biological role of cellular senescence in living organism.

TELOMERE SHORTENING AND SENESCENCE

At the end of eukaryotic chromosomes specialized DNA structure called telomeres stabilize the chromosome ends and prevent chromosomal abnomarilties (6). Telomeres are maintained by a specific enzyme called telomerase, which is not expressed in most normal human somatic cells (10). In these cells, telomere length is sequentially reduced at each cell division due to the end-replication problem. When the telomere reaches a critical length, it is thought to initiate DNA damage response signals and activate p53-dependent checkpoints that contribute to onset of cellular senescence (11). Telomere length therefore functions as a mitotic clock that counts the cell division number in human normal somatic cells. In accord with this model, ectopic expression of the catalytic subunit of telomerase enzyme, hTERT, in some human fibroblasts, halts the erosion of telomeres and prevents the onset of cellular senescence (12, 13). This is suggesting that telomere shortening is indeed a mechanism of onset of cellular senescence. Recent studies have suggested, however, that the altered telomere state rather than loss of telomeric DNA induces cellular senescence (14). The structure of telomeres, the nucleoproteins complex that serves as a protective "cap" for the chromosome end, may undergo changes during cellular proliferation, possibly as a results of telomere-shortening. More recently, unexpected role of endogenous telomerase has been uncovered in normal human somatic cells (15). It has long been believed that normal human somatic cells do not express detectable level of hTERT gene and therefore do not express substantial level of telomerase activity. However, Matsutomi *et al* (2003) has shown that hTERT is actually expressed in normal human fibroblasts and plays important role in maintenance of proper structure of the chromosome end. Indeed, RNA interference (RNAi)-mediated inhibition of endogenous hTERT rapidly induced senescence-like cell cycle arrest in human primary fibroblasts (15). These evidences are also indicating that the "capping" state of telomeres, but not the overall length of telomeres, correlates with the induction of cellulasr senescence.

While telomere shortening occurs in normal human cells, telomere length is maintained either by overexpression of telomerase or by a mechanism known as alternative lengthening of telomeres (ALT) in most human cancer cells (16). Whatever mechanism, it is clear that telomere maintenance is essential for immortality and its control might be a possible therapeutic target in human cancer.

TELOMERE-INDEPENDENT MECHANISMS FOR SENESCENCE

In contrast to human cells, there is no strong evidence in rodent cells that cellular senescence is dependent on telomere erosion (8). Telomere in rodent cells are very long, and most rodent somatic cells have significant level of telomerase expression. Moreover, cellulasr senescence in rodent cells occurs without detectable telomere-shortening (8). How is cellular senescence induced without telomere shortening in rodent cells? This telomere-independent proliferation block may reflect a cell cycle checkpoint response to inappropriate cell culture condition rather than an intrinsic limitation imposed by a cell-division counting mechanism. It is likely therefore that there is a telomere-independent cellular senescence mechanism in higher eukaryotic cells. Indeed, accumulating evidences have demonstrated that various physiological stresses, such as oxidative stress and aberrant mitogenic stimulation, are involved in cellular senescence. For example, it was recently reported that when mouse embryonic fibroblasts (MEFs) are cultured in low (3%) oxygen, rather than normal (20%) oxygen condition, MEFs avoid cellular senescence and continue to proliferate, like a immortalized cells (17). This study also reported that the rapid accumulation of DNA damage was observed in MEFs, when grown in 20% oxygen, but not in 3% oxygen condition. This is consistent with previous observation that human primary fibroblasts are able to undergo more cell divisions prior to cellular senescence when cells were cultured in low (1-3%) oxygen condition (18, 19, 29). These evidences, therefore, suggest that extrinsic physiological stress, such as oxidative stress, is a common factor which induces cellular senescence in both human and rodent cells. Taken together, it is clear that telomere shortening is not a sole mechanism that cause cellular senescence (21, 22).

p16INK4a-RB PATHWAY IN HUMAN CANCER

In higher eukaryotes, the retinoblastoma (RB) and p53 tumors suppressor proteins are crucial gatekeeper of cellular senescence (23, 24). The activities of RB and p53 are tightly regulated by various post-translational modifications, such as phosphorylation, acetylation and ubiquitination (25, 26). RB is thought to impose a block on G1 progression that is alleviated by phosphorylation. In particular, the cyclin-dependent kinases, CDK 2, CDK 4 and CDK 6 play a crtical role in phosphorylation of RB. When RB is phosphorylated by CDKs it loses its function and releases its target, the E2F family transcription factors (E2F1-3), resulting in the initiation of DNA replication (Figure 2)(27, 28). Deregulated activity of the D-type cyclin-dependent

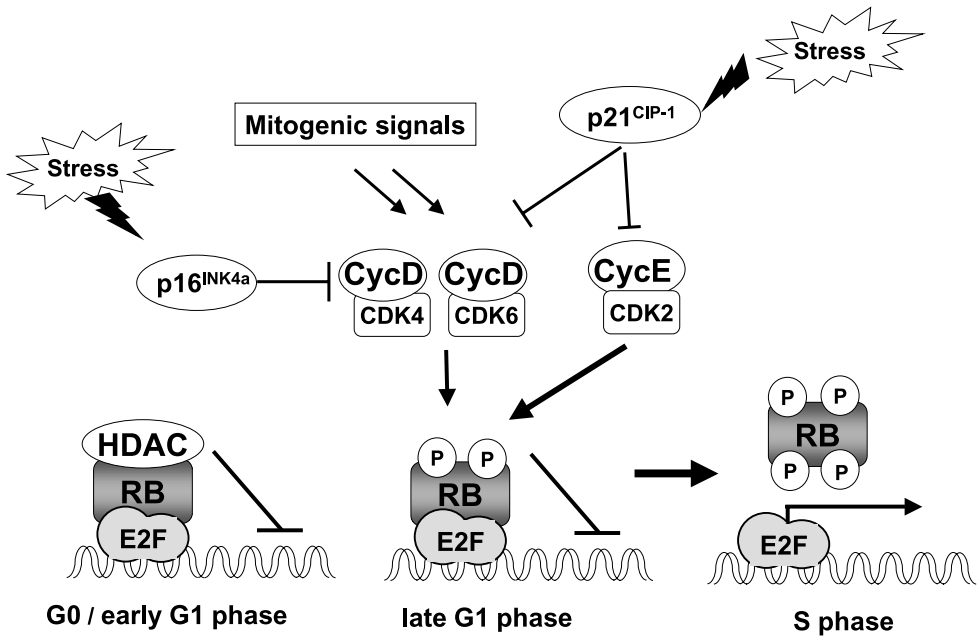

Figure 2. p16INK4a/RB-pathway in cell cycle control
Following mitogenic stimulation, cyclinD and cyclinE in complex with CDK4/6 or CDK2 respectively, sequentially phosphorylate RB thus releasing it from its transcriptionally repressive complexes with E2Fs facilitating S phase progression. Expression of p16INK4a and/or p21Cip1 is induced by distinct stresses, thereby inhibiting CDK activity.

kinases, CDK 4 and CDK 6, is widely observed in various tumor cells, illustrating their importance in controlling cell cycle (29). The cyclin D1 gene is amplified and over-expressed in a variety of human cancers, particularly breast cancer and squamous cell carcinomas, as well as being activated by chromosomal translocation in mantle cell lymphoma. Similarly, CDK4 is amplified in human sarcomas and more recently, a mutant (R24C) which does not bind to its negative regulators, the INK4 family proteins, is found in melanoma (29). More frequently, the $p16^{INK4a}$ gene, a prototype of the INK 4 gene family, is inactivated by various mechanism in a wide range of human cancers (30). All these abnormalities cause deregulation of RB function. The $p16^{INK4a}$/CDK/RB pathway is therefore considered to be an attractive target for therapeutic intervention but more information is needed regarding the detailed mechanisms involved in order to identify the most appropriate strategies for tumor suppression.

We and others have shown that the $p16^{INK4a}$ tumor suppressor gene plays a key role in cellular senescence in human cells. Although the level of $p16^{INK4a}$ is extremely low in normal proliferating cells, the expression of $p16^{INK4a}$ is significantly induced during cellular senescence (31, 32). Moreover, overexpression of $p16^{INK4a}$ results in senescence-like growth arrest, identifying $p16^{INK4a}$ as a strong candidate for mediating cellular senescence (33, 34) So, how is $p16^{INK4a}$ expression induced in senescence? Although we cannot completely preclude the connection between $p16^{INK4a}$ expression and telomere-shortening, its expression is more likely to be regulated by extrinsic stress signaling pathways. This is because accumulation of $p16^{INK4a}$ is observed in both human and rodent senescent cells. Furthermore, Serrano *et al* (1997) have shown that aberrant growth signals provided by constitutive active form (oncogenic) Ras rapidly induce $p16^{INK4a}$ expression without telomere shortening in both human and rodent cells (35)(Figure 1). In remarkable contrast to previous observations using established cell lines (36, 37), introduction of oncogenic Ras into normal primary cells results in the induction of various antiproliferative proteins, including the $p16^{INK4a}$ and p53 tumor suppressors ; the accompanying cell cycle arrest resembles cellular senescence and is termed "premature-senescence" (35)(Figure 1). Normal cells must therefore have a sensor that detects aberrant growth signals, such as too many cell divisions and/or oncogene activation, and that may induce $p16^{INK4a}$ expression.

In rodent cells, targeted inactivation of RB or p53 gene overrides cellular senescence (38, 39). Moreover, triple knock-out MEFs lacking all Rb-family genes (Rb, p107 and p130) do not senesce despite the presence of high levels of p53, suggesting that RB-family proteins play a role downstream of the p53 pathway in mouse cell senescence (40, 41). In human cells, however, inactivation of both RB and p53 is required to override cellular senescence in most cell types, suggesting that p53 and RB have overlapping, but distinct, roles in human cell senescence (23, 24).

TRANSCRIPTIONAL REGULATION OF $p16^{INK4a}$ EXPRESSION

Previous experiments with Ras mutants, which are unable to activate one or another effector pathway of Ras signaling, have identified that the Ras/MEK signal transduction pathway is crucial for Ras-induced cellular senescence (42, 43). We have shown that $p16^{INK4a}$ expression is controlled by the Ets 1 and Ets 2 (Ets1/2) transcription factor, which are down-stream mediators of the Ras/Raf/MEK-pathway (44). Normally, the Ets 1/2 transcription factor is phosphorylated by MAP kinase at certain points in the cell cycle. Id1, a transcriptional inhibitor, counter-balances this activity by binding to and inactivating Ets 1/2. In replicative senescence, which is provoked by cumulative cell divisions, expression levels of Ets 1 increase whilst Id 1 levels decline. Thus Ets1 likely to induce $p16^{INK4a}$ expression aided by the concomitant down-regulation of Id1. In premature senescence, which is provoked by oncogenic Ras, Ets1/2 is constitutively activated by oncogenic Ras signals. This aberrant activation overrides the steady state and results in induction of $p16^{INK4a}$ expression and premature senescence. This model is further supported by recent study using dermal fibroblasts, Q34 cells, from an individual carrying biallelic mutation in $p16^{INK4a}$ gene (45). Both mutations alter the aminoacids sequence of $p16^{INK4a}$ and functionally impaired the activity of $p16^{INK4a}$ protein. In contrast, only one of the mutations affects the sequence of $p14^{ARF}$ protein, causing an apparently innocuous changes near its carboxy terminus (Figure 3). Ectopic expression of oncogenic Ras or its downstream effectors Ets1and Ets 2 failed to induce premature senescence in Q34 fibroblasts, although the similar levels of oncogenic ras or Est1/2s expression efficiently induced premature senescence in normal (wild type) human fibroblasts (45). This is suggesting that $p16^{INK4a}$ is a critical downstream mediator for Ras/Ets-pathway in premature senescence. This data also imply that p 16^{INK4a}, but not $p14^{ARF}$, assumes the principal role in Ras-induced premature senescence in human fibroblasts. It is,

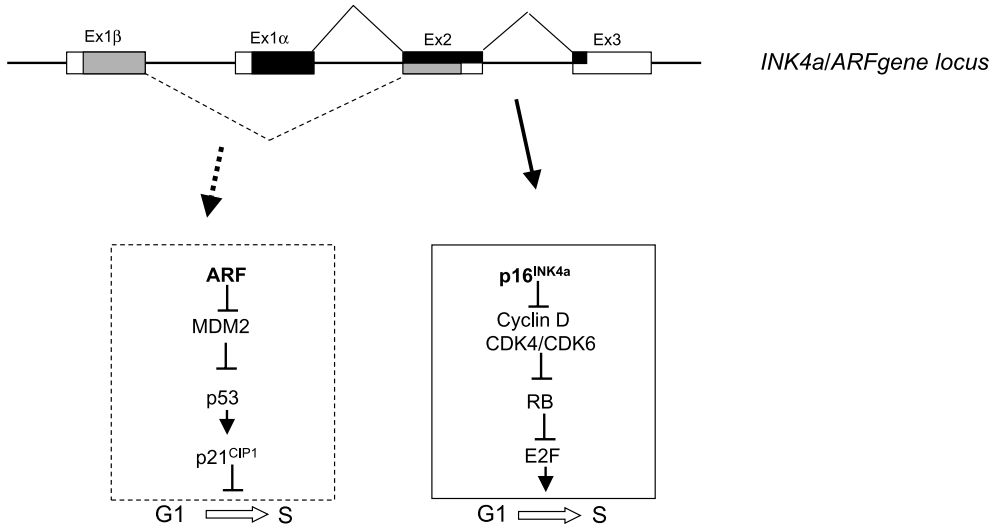

Figure 3. INK 4 a/ARF tumor suppressor gene locus regulates RB/p53 pathways
INK4a/ARF locus encodes two proteins regulating RB or p53 tumor suppressor pathways. Although $p14^{ARF}$ does not act as a CDK inhibitor, $p14^{ARF}$ induces $p21^{Cip1}$, a CDK inhibitor, through activating p53-pathway.

however, important to note that other factors are involved in this process. In particularly, chromatin remodeling factors such as SNF5 and BMI-1 are likely to be associated with Ras/Ets/$p16^{INK4a}$-pathway (46-49).

$p16^{INK4a}$ VERSUS $p14^{ARF}$ IN HUMAN CANCER

The INK4a/ARF gene locus has the unusual capacity to encode two structurally distinct proteins, $p16^{INK4a}$ and $p14^{ARF}$ ($p19^{ARF}$ in mouse cells), by reading a shared second exon in different translational reading frames (Figure 3). Although $p16^{INK4a}$ binds directly to and inhibits the activity of CDK4 and CDK6, $p14^{ARF}$ binds directly to MDM2, but not CDKs, resulting in the stabilization and the activation of p53-target genes (30). In mouse model system, $p19^{ARF}$ plays an important protective role from tumorigenicity whereas $p16^{INK4a}$ loss causes only a limited induction of tumors (50). In MEFs, the levels of $p19^{ARF}$ is significantly induced in both oncogenic Ras induced senescence and in replicative senescence (35). However, this is not the case in human primary fibroblasts, suggesting the differential importance of INK 4 a/ARF gene locus in human and mouse (51, 52). To directly assess the individual contribution of $p16^{INK4a}$ and $p14^{ARF}$ genes to tumor suppressor pathways in human, Voorthoeve and Agami (2003) have specifically blocked $p16^{INK4a}$ and / or $p14^{ARF}$ gene expression using RNAi mediated-gene silencing technology (53). Voorhoeve and Agami has shown that suppression of $p16^{INK4a}$ expression, but not $p14^{ARF}$ expression, synergize with p53 loss to accelerate growth and causes transformation. These results are consistent with the notin that $p16^{INK4a}$ is acting as a tumor suppressor gene in human cancer.

INACTIVATION OF $p16^{INK4a}$-RB PATHWAY IN HUMAN CANCER

It is evident that the expression of $p16^{INK4a}$ gene is dramatically reduced in several human cancers due to the hyper-methylation of $p16^{INK4a}$ gene promoter (54). Recent studies, however, indicated that a different mechanism blocking the $p16^{INK4a}$ gene expression is existing in certain types of human cancers (55). The latent membrane protein 1 (LMP 1) oncoprotein of Epstein-Barr virus (EBV) is often present in EBV-associated proliferative diseases and is critical for the immortalizing and transforming activity of EBV. Unlike other DNA tumor virus oncoproteins which possess immortalizing activity, LMP1does not bind to RB, but instead blocks the expression of the $p16^{INK4a}$ gene (55). However, it has been unclear how LMP1 represses the $p16^{INK4a}$ gene expression. We have now found that LMP 1 promotes the CRM1-dependent nuclear export of Ets 2, thereby reducing the level of $p16^{INK4a}$ gene expression (56). We further demonstrate that LMP1 also blocks the function of repressive E2Fs (E2F4 and E2F5), which primarily repress the E2F-target gene expression, through promoting their nuclear export in a CRM1-dependent manner (56). As E2F4 and E2F5 are essential downstream mediators for a $p16^{INK4}$ a-induced cell cycle arrest (57), these results indicate that the action of LMP 1 on nuclear export

has two effects on the $p16^{INK4a}$-RB pathway : (i) repression of $p16^{INK4a}$ expression and (ii) blocking the downstream mediator of the $p16^{INK4a}$-RB pathway. These results reveal a novel activity of LMP1 and increase an understanding of how viral oncoproteins perturb the $p16^{INK4a}$-RB pathway leading to cancer. Understanding the mechanisms of LMP1-induced nuclear export would help us to find a way of specific suppression of EBV associated cancers.

CONCLUDING REMARKS

It has recently become evident that cellular senescence, the final resting place of the cell, is a state induced by a variety of distinct physiological stresses including oncogene activation. These stresses activate cell cycle checkpoint response such as induction of $p16^{INK4a}$ and cause permanent growth arrest. Although we still do not have definitive evidence that cellular senescence occurs *in vivo*, physiological stresses including oncogene activation, rather than cumulative cell divisions, could turn out to be a physiologically relevant senescence trigger. The identification of senescent cells, especially in the context of the human body, will provide valuable new insights into the development of cancer and open up new possibilities of its control.

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