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Review

The ARF-p53 senescence pathway in mouse and human cells

R. Wadhwa[1], T. Sugihara[2], K. Taira[1] and S.C. Kaul[1]

[1]National Institute of Advanced Industrial Science and Technology (AIST),Tsukuba, Ibaraki, Japan and

[2]Department of Radiobiology, Institute for Environmental Sciences, Kamikita, Aomori, Japan

Summary. Mouse and human cells have most frequently been used for studies that have led to the elucidation of various molecular pathways involved in senescence. The ARF–p53 pathway has been assigned as one of the major protagonists in these phenomena. ARF is an alternative reading frame protein encoded along with $p16^{INK4A}$ by the INK4a locus on human chromosome 9p21 and the corresponding locus on mouse chromosome 4. Whereas the mouse ARF (p19ARF) consists of 169 amino acids, the human ARF (p14ARF) consists of 132 amino acids, truncated at the C-terminus. Molecular studies on the regulation of ARF activity by its binding partners have revealed that mouse ARF protein, but not human ARF protein, interacts with a cytoplasmic protein, Pex19p. This interaction of mouse ARF with Pex19p results in its milder p53 activation function in mouse cells as compared to human cells and thus accounts, at least in part, for the weaker tumor surveillance and frequent immortalization of mouse cells.

Key words: p19ARF, p14ARF, Pex19p, p53-regulation, Senescence

Cellular senescence

Normal somatic cells have limited proliferative capacity and eventually reach a state of a permanent growth arrest. This viable and metabolically active stage is the most consistent manifestation of cellular senescence and is referred to as replicative senescence. In the last two decades, molecular and genetic studies have unraveled many aspects of cellular senescence. These include the understanding of the role of tumor suppressor genes, epigenetic controls and the role of the telomeres in the control of normal cell proliferation. The present understanding of cellular aging has the following major components: i) it is an inevitable outcome of gene activities (longevity - assuring genes) that are essential for maintaining the order, integrity and normal functions, ii) since living systems are not equipped with perfect maintenance and repair mechanisms, these confront accumulation of genetic, cellular and extra-cellular damages; iii) longevity-assuring functions, but not their by-product accumulation of damage, are genetically programmed and are monitored by gene activities including tumor suppression mechanism(s) such as activities of negative regulators of cell cycle and telomere shortening; and iv) abrogation of one or more tumor suppressor gene functions and maintenance of repair mechanisms is sufficient to escape senescence, at least *in vitro* (Wadhwa et al., 2003a).

Offprint requests to: Sunil C. Kaul, National Institute of Advanced Industrial Science & Technology (AIST), Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan. e-mail: s-kaul@aist.go.jp

Why is the mouse used for aging studies?

The mouse is the most popular model among other widely studied genetically malleable species due to: 1) Its evolutionarily close relationship to human. Mouse genome sequencing has predicted the presence of around 30,000 genes, lacking only 300 genes (1%) when compared to human. 2) The availability of wild, inbred and mixed inbred strains which make the genetic studies feasible. 3) The technical ability to manipulate the mouse germline through transgenic and knockout methods and thus there is availability of spontaneous and engineered mouse mutants. 4) The technical ability to culture mouse cells from a variety of lineages. 5) The short lifespan of 2-3 years which allows the performance of aging studies within a reasonable length of time. 6) The fact that development, genetics, biology, pathology and aging of various strains of mice have been well characterized. 7) The fact that unlike worms and flies, whose somatic cells are virtually all post-mitotic as adults, mice have mitotic compartments of self-renewing tissue stem cells that play an important role in organism aging by maintaining organ homeostasis.

Replicative senescence in mouse and human cells

Mouse cells show a period of rapid growth, followed by a slowing down and cessation of cell proliferation which is accompanied by an elevated expression of

negative cell-cycle regulators including CDK inhibitors, $p16^{INK4A}$, $p21^{WAF1}$, $p19^{ARF}$ and p53. Rare variants arise from the late-phase slow cultures and switch to fast proliferation leading to establishment of immortal lines (Sherr and DePinho, 2000; Wright and Shay, 2002). Genetic analysis has identified the loss of p53 function and chromosomal instability as prime changes in these cells (Todaro et al., 1965; Harvey and Levine, 1991). Indeed, cells from p53-null or ARF-null mice were shown to maintain a high proliferation potential and immortalize in culture without showing any signs of senescence. Therefore, in mouse cells, disruption of p53 function either by mutation or by its functional inactivation by perturbance of its regulators (ARF and MDM2) is sufficient to make them immortalize (Donehower, 2002). Both $p19^{ARF}$ and $p16^{INK4A}$ are coded by the same gene locus, and genetic manipulation of individual exons of the CDKN2A locus to obtain silencing of one transcript at a time has further documented contributions of $p16^{INK4A}$ and $p19^{ARF}$ in mouse cell senescence. Abrogation of any one of these facilitates immortalization and subsequent carcinogenesis of cells (Kamijo et al., 1997; Krimpenfort et al., 2001; Sharpless et al., 2001). Primary mouse embryonic fibroblasts (MEFs), when exposed to oncogenic Ras, express senescence-specific cell-cycle inhibitors and undergo premature senescence (Serrano et al., 1997). In contrast, the immortalized cells lacking p53 or ARF are efficiently transformed (Kamijo et al., 1997). These studies have suggested that senescence operates as a tumor-suppressor mechanism in mouse cells wherein the $p19^{ARF}$-p53-$p21^{WAF1}$ pathway acts as a main player.

Human cells have a more extended proliferation potential than mouse cells but rarely become adapted to continuous proliferation and establishment into permanent cell lines. They are genetically more stable and more resistant to oncogene-mediated transformation than mouse cells. Experiments designed to dissect the role of p53 and pRB have suggested that in contrast to the mouse system, in which p53 plays a major role where its inactivation is sufficient to escape from mortality, the human cells have more rigid constrains that involve the participation of p53 and pRB tumor-suppressor pathways. Inactivation of either or both of these proteins (e.g. by SV40 large T antigen) is sufficient to confer escape from senescence; inactivation of both proteins has a co-operative effect on lifespan extension (Shay et al., 1993; Rogan et al., 1995; Bond et al., 1999). However, human cells with extended lifespan can only establish into permanent cell lines with additional genetic events culminating in activation of telomerase or ALT (alternative lengthening of telomeres) which leads to maintenance of otherwise eroding telomeres (Duncan and Reddel, 1997; Duncan et al., 2000; Maser and DePinho, 2002; Stewart and Weinberg, 2002). Furthermore, a second product of the early region, the small t antigen (stAg), is essential for the transforming potential for human, but not mouse cells. stAg perturbs one or more isoforms of the widely acting serine/threonine phosphatases (PP2A); the PP2A pathways perturbed by stAg that are essential for human cell transformation remain to be defined (de Ronde et al., 1989).

Mouse and human show major differences in involvement of telomeres (nucleoprotein structures consisting of GT-rich tandem repeats of sequence (TTAGGG) and their binding proteins) in senescence. The telomeres are specialized structures that protect the ends of linear chromosomes and maintain the chromosomal integrity by preventing their end-to-end fusion. Due to the semi-conservative mode of replication telomeres face an end replication problem. RNA–primed DNA synthesis of the lagging strand results in a terminal gap after degradation of the most distal primer. The single-stranded GT-rich 3' overhang at each telomere is degraded by exonuclease activity leading to progressive shortening of the telomeric DNA template with each round of DNA replication serving as a mitotic clock to indicate the number of times the cell has divided and can be expected to divide. In the laboratory mouse, an inbred strain of *Mus musculus*, telomeres are much longer (~60 kb) than those in human cells (~12 kb). Although the mouse cells begin their replication ex vivo with extremely long telomeres, they undergo replicative arrest after only 15-20 population doublings, well before the detectable telomere shortening. Thus, although mouse and human senescent cells share many morphological and biochemical features, the very long telomeres in mouse make it unlikely that telomere shortening provides a signal for activation of the senescence program (Wadhwa et al, 2003a).

Tumor suppressor (p53 and pRB)-driven cellular senescence in mouse and human

Hypo-phosphorylated pRB binds and inactivates the E2F family of transcriptional factors and inhibits the progression of the cell cycle. Phosphorylation of pRB by cyclin-CDK complexes leads to the release of E2F and the entry of cells to the S-phase. Thus, cyclin-CDK complexes play a major role in the execution of cell-cycle proliferation or arrest. The activity of cyclin-CDK complexes is inhibited by two classes of inhibitors, $p21^{WAF1}$ (a downstream effector of p53) and $p16^{INK4A}$. The latter is coded from an INK4a (MTS1, CDKN2) locus that encodes another unrelated-tumor suppressor protein, p19ARF, an alternative reading frame protein that acts upstream of p53. Both of these proteins have been shown to play roles in replicative- and Ras-induced premature senescence of primary cells (Quelle et al., 1995; Serrano et al., 1996; Kamijo et al., 1997).

One of the ways p19ARF and its human homologue p14ARF activate p53 function is by mechanisms that involve activation of p53 by restraining its anatgonist, MDM2 (Stott et al., 1998; Zhang et al., 1998; Honda and Yasuda, 1999; Tao and Levine, 1999; Weber et al., 1999). ARF can also function by pathways independent of p53 and MDM2 (Eymin et al., 2001; Weber et al.,

2000). The activity of ARF is regulated by its homo-oligomerization (Menendez et al., 2003) and binding to various other proteins including E2F family members (Martelli et al., 2001), spinophilin (Vivo et al., 2001), topoisomerase I (Karayan et al., 2001), mdmX (Jackson et al., 2001) and Pex19p/HK33/HsPXF (Sugihara et al., 2001), cyclin G1 (Zhao et al., 2003), p120 (E4F) (Rizos et al., 2003) and CARF (Hasan et al., 2002; Wadhwa et al., 2003b). The functional relevance of most of these interactions during cellular senescence, tumor suppression or apoptosis remains poorly defined. We have identified the interaction of p19ARF with a cytoplasmic protein (Pex19p) and have demonstrated (described below) that such an interaction is specific to the mouse ARF protein only.

Functional differences in the ARF-p53 pathway in mouse and human cells

To investigate the molecular mechanism of ARF-function, we identified its binding partners by yeast two-hybrid screen (Sugihara et al., 2001) using p19ARF as a bait. One of the strongly interacting proteins was identified as farnesylated protein Pex19p/HK33/HsPXF (essential for peroxisomal biogenesis) (Braun et al., 1994; Kammerer et al., 1997; Gotte et al., 1998). The interactions of p19ARF and Pex19p were confirmed by co-immunoprecipitation assays and it was found that these two proteins interact in the cell cytoplasm (Fig. 1A, Sugihara et al., 2001). Noticeably, overexpression of Pex19p in cells resulted in cytoplasmic retention of the p19ARF protein (Fig. 1A). Since p19ARF was shown to neutralize the p53 antagonist (MDM2) in the nucleus, these results predicted that p19ARF-Pex19p interactions may cause inactivation of the p19ARF function. High levels of MDM2 protein lead to p53 degradation resulting in its inactivation. Such an exclusion of $p19^{ARF}$ from the nucleus and the inactivation of p53 function constitutes a novel mechanism of functional dampening of the $p19^{ARF}$-p53 pathway. We have tested this

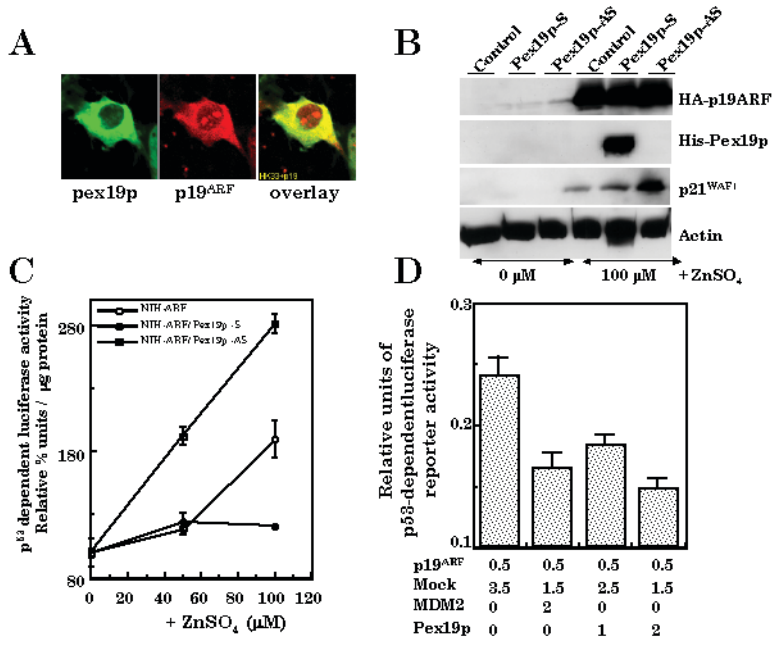

Fig. 1. Pex19p interacts withp19^{ARF} and inactivates its p53 activation function. **A.** Co-immnostaining of Pex19p and p19^{ARF} in the cell cytoplasm. **B.** Decreased amount of p21^{WAF1} in cells transfected with p19^{ARF} and Pex19p. The cells transfected with p19ARF and Pex19p (antisense) show higher levels of p21^{WAF1}. Actin is used as a loading control. **C.** Accelerated p53 activity in cells transfected with p19^{ARF} and Pex19p-antisense. **D.** Attenuation of p53 function in cells transfected with p19^{ARF} and Pex19p. The decrease in p53 activity caused by Pex19p is comparable to that caused by MDM2. (Experimental and technical details are described in Sugihara et al., 2001).

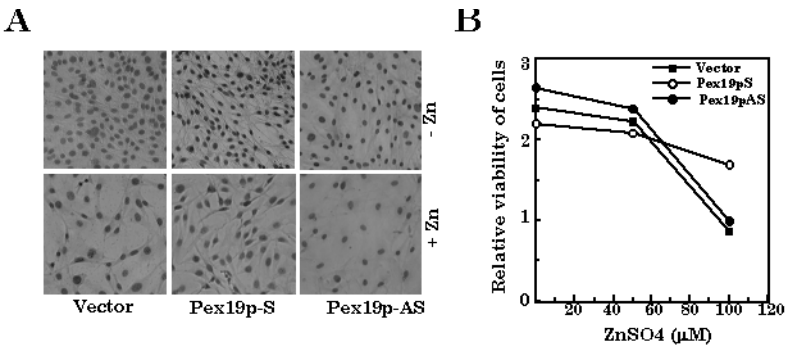

Fig. 2. The effect of Pex19p in cell proliferation and viability. **A.** Accelerated growth arrest of cells caused by expression of p19^{ARF} and Pex19p (antisense). **B.** Cells expressing p19^{ARF} and Pex19p (sense or antisense) show a comparable mitochondrial function (viability). (Experimental and technical details are described in Sugihara et al. 2001).

hypothesis by overexpression of Pex19p and its neutralization by an antisense expression. Whereas the level of expression of the p53–dependent reporter and an endogenous downstream effector, p21WAF1, was suppressed in Pex19p-overexpressing cells, its level was elevated when Pex19p was neutralized (Fig. 1B,C) (Sugihara et al., 2001). An inactivation of the p53 function by overexpression of Pex19p was compared with the cells overexpressing MDM2 by employing p53-dependent reporter assays (Fig. 1D). The reduction in p53 activity by overexpression of Pex19p and MDM2 was found to be similar suggesting that the functional dampening of $p19^{ARF}$ by Pex19p is significant. Furthermore, the neutralization of Pex19p by antisense led to an accelerated activation of the $p19^{ARF}$ function and p53-$p21^{WAF1}$-mediated cell-cycle arrest that resembled cellular senescence phenotype (Fig. 2) (Sugihara et al., 2001). NIH 3T3 cells overexpressing antisense Pex19p showed accelerated senescence-like growth arrest induced by $p19^{ARF}$. We also analyzed the viability of cells transfected with (i) ARF, (ii) ARF and Pex19p-sense and (iii) ARF and Pex19p-antisense encoding expression plasmids. Although the cells showed significant differences in their proliferation, their viability was not affected, as was measured by mitochondrial function (Fig. 2B). These findings suggested that the activation of the ARF function by neutralization of Pex19p results in a state of growth arrest in which cells remain viable and metabolically unaltered.

Studies to define the binding domain of ARF and Pex19p assigned a carboxy-terminus region of p19ARF as its binding domain to Pex19p (Fig. 3A) (Wadhwa et al., 2002). ß-gal reporter assay (dependent on the interactions of two proteins) was performed on yeast cells transformed with Pex19p and full-length p19ARF and its various deletion mutants or p14ARF. The full p19ARF protein and its carboxy-terminal deletion mutants with a minimum of 41 amino acids were positive for interactions with Pex19p. Thus the Pex19p-binding domain of $p19^{ARF}$ was assigned to its carboxy-terminus 41 amino acid residues (129-169 aa.) (Fig. 3A, Wadhwa et al., 2002). Notably, The mouse ARF (p19ARF) consists of 169 amino acids and the human ARF (p14ARF) consists of 132 amino acids; truncated by 37 amino acids at the carboxy-terminus (Bates et al., 1998). The human ARF did not show interactions with Pex19p in either yeast two-hybrid or in co-immunoprecipitation assays (Fig. 3A,B). These data indicated that p14ARF, due to the lack of its interactions with Pex19p, may rapidly translocate to the nucleus without any halt in the cytoplasm which is what occurs in the case of p19ARF (Fig. 3C, Wadhwa et al., 2002). We then compared the subcellular localization of exogenous mouse and human ARF proteins in an

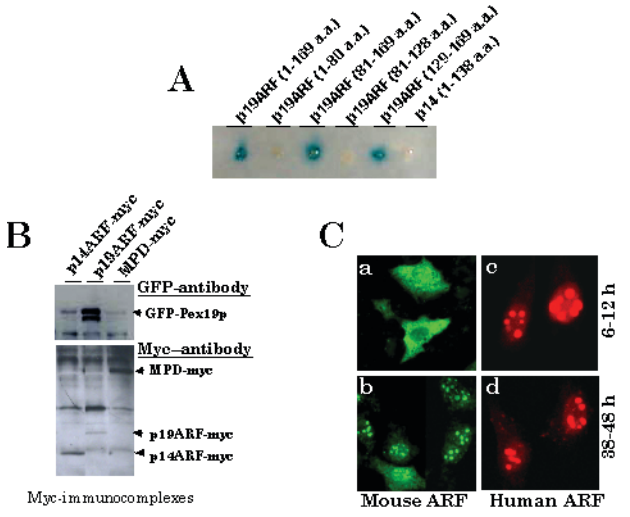

Fig. 3. p19ARF, but not p14ARF, binds to Pex19p. **A.** Interaction of ARF and Pex19p proteins as analyzed by the yeast two-hybrid reporter assay. Blue-colored colonies indicate interaction of the indicated ARF protein with Pex19p. **B.** Co-immunoprecipitation of Pex19p with $p19^{ARF}$,but not with $p14^{ARF}$. **C.** Cytoplasmic localization of $p19^{ARF}$, but not of $p14^{ARF}$. (Experimental and technical details are described in Wadhwa et al., 2002).

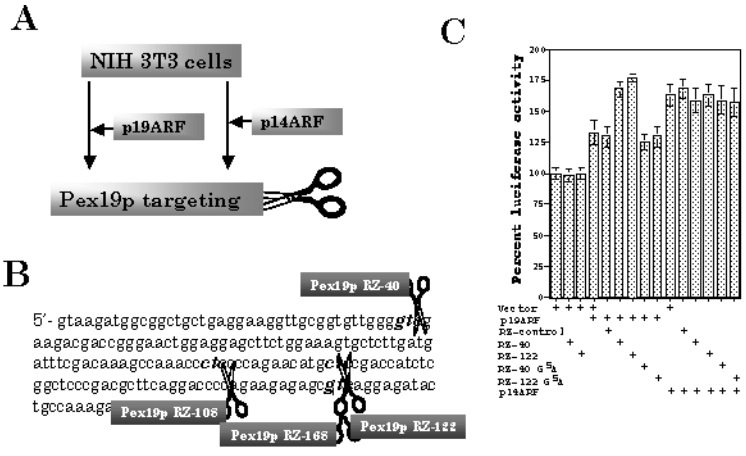

Fig. 4. Targeting of Pex19p improves $p19^{ARF}$ but not $p14^{ARF}$ function. **A.** An experimental design. **B.** Target sites on Pex19p for hammerhead ribozymes. **C.** p53-dependent reporter assay in cells transfected with $p19^{ARF}$/$p14^{ARF}$- and Pex19p-targeting ribozymes. (Experimental and technical details are described in Wadhwa et al., 2002).

identical cellular background. In time-course experiments, $p19^{ARF}$ localized first in the cytoplasm and subsequently translocated to the nucleus and then to the nucleolus (Fig. 3C) (Wadhwa et al., 2002). In contrast, p14ARF was visible in the nucleus even at the earliest time points (6 h) (Fig. 3C). In agreement with these data, p14ARF had a stronger p53-activation function and led to stronger suppression of colony-forming efficiency when compared to the $p19^{ARF}$ (Wadhwa et al., 2002). ARF proteins driven by a metal-responsive promoter were stably transfected into NIH 3T3 cells. The G418-selected clones were assayed for colony-forming efficiency with or without ARF expression (induced by addition of 100 μM $ZnSO_4$ into the medium). Whereas the expression of $p14^{ARF}$ resulted in an 87% reduction in colony-forming efficiency, the expression of $p19^{ARF}$ caused a 45% reduction (Wadhwa et al., 2002). These results clearly showed that the tumor suppressor activity of p14ARF is much stronger than $p19^{ARF}$. Together with the finding that Pex19p holds $p19^{ARF}$ but not $p14^{ARF}$ in the cytoplasm, it was clear that p14ARF, because of its interactions with Pex19p gets quickly translocated to the nucleus and thus possesses stronger activity.

Functional inactivation of $p19^{ARF}$ by Pex19p was further examined by targeting Pex19p expression by hammerhead ribozymes (Wadhwa et al., 2002). Cells were transfected with either p19ARF or p14ARF, and the Pex19p-specific ribozyme expressing plasmid was assayed for p53-dependent luciferase-reporter activity. As expected, p19ARF or p14ARF resulted in an upregulation of p53-dependent reporter activity (Fig. 4). Co-expression of Pex19p ribozymes (Rz-40 and Rz-122) resulted in a further enhancement of p53 activity by 40%; the inactive versions of these ribozymes were neutral (Fig. 4) (Wadhwa et al., 2002). Most notably, these ribozymes did not affect p14ARF-dependent p53 transcriptional-activation function. Taken together, these results demonstrated that Pex19p interacts with the mouse ARF protein and inactivates its function; human ARF, by lacking the Pex19p binding region, escapes from such inactivation making it a stringent tumor suppressor in human cells.

The ARF locus upregulates p53 activity to induce cell-cycle arrest, sensitizes cells to apoptosis by inhibiting MDM2 activity and also causes p53-independent apoptosis. In contrast to p14ARF, ectopic expression of the carboxy-terminus region of p19ARF, which is unique to the mouse ARF, induces p53-independent apoptosis in mouse fibroblasts. Taken together with our study it is suggested that the carboxy-terminal region of p19ARF dampens p53 activity (Sugihara et al., 2001) on one hand and mediates p53-independent apoptosis on the other (Matsuoka et al., 2003). ARF is a major tumor suppressor and is involved in cellular senescence. Such a difference in its activity in mouse and human cells may explain a molecular basis of firm control of cellular senescence, a barrier to immortalization and better tumor surveillance in human cells. These studies argue that although human and mice share a common set of protein ingredients, there are several important differences in their regulation that in turn lead to their different senescing, immortalization and stress-response profiles.

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