[CANCER RESEARCH 60, 3689–3695, July 15, 2000]

Special Lecture

The Pezcoller Lecture: Cancer Cell Cycles Revisited

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Abstract

Genetic lesions that disable key regulators of G_1 phase progression in mammalian cells are present in most human cancers. Mitogen-dependent, cyclin D-dependent kinases (cdk4 and cdk6) phosphorylate the retinoblastoma (Rb) tumor suppressor protein, helping to cancel its growth-inhibitory effects and enabling E2F transcription factors to activate genes required for entry into the DNA synthetic phase (S) of the cell division cycle. Among the E2F-responsive genes are cyclins E and A, which combine with and activate cdk2 to facilitate S phase entry and progression. Accumulation of cyclin D-dependent kinases during G_1 phase sequesters cdk2 inhibitors of the Cip/Kip family, complementing the effects of the E2F transcriptional program by facilitating cyclin E-cdk2 activation at the G_1-S transition. Disruption of "the Rb pathway" results from direct mutational inactivation of Rb function, by overexpression of cyclin D-dependent kinases, or through loss of $p16^{INK4a}$, an inhibitor of the cyclin D-dependent kinases. Reduction in levels of $p27^{Kip1}$ and increased expression of cyclin E also occur and carry a poor prognostic significance in many common forms of cancer. The ARF tumor suppressor, encoded by an alternative reading frame of the *INK4a-ARF* locus, senses "mitogenic current" flowing through the Rb pathway and is induced by abnormal growth promoting signals. By antagonizing Mdm2, a negative regulator of the p53 tumor suppressor, ARF triggers a p53-dependent transcriptional response that diverts incipient cancer cells to undergo growth arrest or apoptosis. Although ARF is not directly activated by signals that damage DNA, its loss not only dampens the p53 response to abnormal mitogenic signals but also renders tumor cells resistant to treatment by cytotoxic drugs and irradiation. Lesions in the p16 — cyclin D-CDK4 — Rb and ARF — Mdm2 — p53 pathways occur so frequently in cancer, regardless of patient age or tumor type, that they appear to be part of the life history of most, if not all, cancer cells.

Introduction

"The greatest single achievement of nature to date was surely the invention of the molecule of DNA. We have had it from the very beginning, built into the first cell to emerge, membranes and all, somewhere in the soupy water of the cooling planet three thousand million years or so ago. All of today's DNA, strung through all of the cells of the earth, is simply an extension and elaboration of that first molecule. In a fundamental sense, we cannot claim to have made progress, since the method used for growth and replication is essentially unchanged" (1).

As the late Lewis Thomas implied, the principle task of the cell division cycle is to replicate DNA (without errors during S phase) and to segregate the duplicated chromosomal DNA equally to two daughter cells [during mitosis (or M phase)] (Fig. 1). In addition to the molecular regulators that drive these processes, a monitoring circuitry ensures that S phase is completed before mitosis begins and *vice versa*. Early embryonic cell cycles exhibit rapidly alternating S and M phases without gap phases between them. This suggests that the gap phases seen in somatic cell cycles–G_1 separating the M and S phases, and G_2 separating the S and M phases–are not strictly essential for the correct operation of the cell cycle engine. It is intriguing to reflect on this point, because many of the G_1 phase regulators that prove so important in accelerating or braking the cell cycle engine of mammalian cells are encoded by nonessential genes, whose elimination from the germ line needs not lead to deleterious effects on organismal development.

G_1 phase is the interval in which cells respond to extracellular cues that ultimately determine whether cells will make the decision to replicate DNA and divide or, alternatively, to exit the cell cycle into a quiescent state (G_0). Once cells make the decision to begin DNA replication, they are irreversibly committed to complete the cycle, and the time late in G_1 phase at which this decision is made was designated the "restriction point" by Arthur Pardee (reviewed in Ref. 2; Fig. 1). When cells are stimulated by growth factors to enter the cycle from G_0, they generally require continuous mitogenic stimulation to be driven to the restriction point, after which mitogens can be withdrawn and cells will enter S phase and complete the cycle in their absence. Conversely, antiproliferative compounds, such as cytokines like transforming growth factor-β or drugs such as rapamycin, can only arrest the proliferation of cells that are progressing through G_1 phase but have not yet reached the restriction point. In mammalian cells, progression through the restriction point involves a series of events that lead to, but are distinct from, the firing of replication origins and that temporally precede the G_1-S transition by several hours (Fig. 1).

G1 Cyclins and cdks

In general, cell cycle transitions are controlled by cdks.[2] These holoenzymes contain both regulatory (cyclin) and catalytic (cdk) subunits but likely exist as higher order complexes that include additional proteins (see below). Restriction point control is mediated by two families of enzymes, the cyclin D- and E-dependent kinases. The D-type cyclins (D1, D2, and D3; Refs. 3–5) interact combinatorially with two distinct catalytic partners (cdk4 and cdk6; Refs. 6 and 7) to yield at least six possible holoenzymes that are expressed in tissue-specific patterns. Whereas cdk4 and cdk6 are relatively long-lived proteins, the D-type cyclins are unstable, and their induction, synthesis, and assembly with their catalytic partners all depend upon persistent mitogenic signaling. In this sense, the D-type cyclins act as growth factor sensors, forming active kinases in response to extracellular cues (reviewed in Ref. 8).

The mitogen-dependent accumulation of the cyclin D-dependent kinases triggers the phosphorylation of Rb, thereby helping to cancel its growth-repressive functions (6, 9, 10). Rb represses the transcription of genes whose products are required for DNA synthesis. It does so by binding transcription factors such as the E2Fs (reviewed in Ref. 11) and recruiting repressors such as histone deacetylases (12–14) and chromosomal remodeling SWI/SNF complexes (15) to E2F-responsive promoters on DNA. However, Rb phosphorylation by the G_1

Received 4/18/00; accepted 5/8/00.

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[2] The abbreviations used are: cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; ARF, alternative reading frame; MEF, mouse embryo fibroblast.

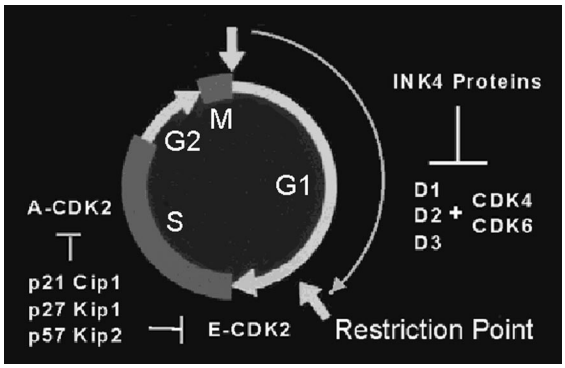

Fig. 1. G1 cdks and cdk inhibitors. Somatic cell cycles consist of alternating DNA synthetic (*S*) and mitotic (*M*) phases, separated by gap phases (*G1* and *G2*) as indicated. Mammalian cells respond to extracellular mitogens and antiproliferative cytokines from the time that they exit mitosis (*vertical arrow* at *top*) until they reach the restriction point, after which they can complete the cell division cycle in the absence of extracellular growth factors. Cyclin D-dependent kinases accumulate in response to mitogenic signals and initiate the phosphorylation of Rb, a process that is completed by cyclin E-cdk2. Once cells enter S phase, cyclin E is degraded and cyclin A enters into complexes with cdk2. INK4 proteins oppose the activities of the various cyclin D-dependent kinases, whereas Cip/Kip proteins specifically inhibit cyclin E-cdk2 and cyclin A-cdk2.

cdks disrupts these interactions (15, 16), enabling untethered E2Fs to function as transcriptional activators (Fig. 2). Apart from a battery of genes that regulate DNA metabolism, E2Fs induce the cyclin E and A genes. Cyclin E enters into a complex with its catalytic partner cdk2 (17–20) and collaborates with the cyclin D-dependent kinases to complete Rb phosphorylation (Fig. 2; Refs. 21–26). This shift in Rb phosphorylation from mitogen-dependent cyclin D-cdk4/6 complexes to mitogen-independent cyclin E-cdk2 accounts in part for the loss of dependency on extracellular growth factors at the restriction point. Cyclin E-cdk2 also phosphorylates substrates other than Rb, and its activity is somehow linked to replication origin firing (27, 28). The activity of the cyclin E-cdk2 complex peaks at the G_1-S transition, after which cyclin E is degraded and replaced by cyclin A (Fig. 1).

Cdk Inhibitors: The Cip/Kip Family

The actions of cdk2 are opposed by the *Cip/Kip* family of polypeptide inhibitors that includes $p21^{Cip1}$ (29–31), $p27^{Kip1}$ (32–34), and $p57^{Kip2}$ (Refs. 35 and 36; reviewed in Ref. 37; Fig. 1). In quiescent cells, the levels of $p27^{Kip1}$ are generally high. However, as cells enter cycle and accumulate cyclin D-dependent kinases, the Cip/Kip proteins are sequestered in complexes with cyclin D-dependent cdks (Fig. 2). Although it was initially assumed that the Cip/Kip proteins would inhibit both cdk4/6 and cdk2, we now recognize that the Cip/Kip-bound cyclin D-dependent enzymes remain catalytically active (38–41). Even more surprisingly, it turns out that Cip/Kip proteins are required for the assembly of the active cyclin D-dependent holoenzymes (Refs. 40 and 41; Fig. 2). In cycling cells, virtually all of the $p27^{Kip1}$ molecules remain associated with cyclin D-cdk complexes. However, mouse embryo fibroblasts taken from animals lacking both the *Kip1* and *Cip1* genes–$p57^{Kip2}$ is not synthesized in these cells–express no detectable cyclin D-dependent kinase activity and still have relatively unperturbed cell cycle transit times (41). In this setting, the levels of cyclin E-cdk2 activity are greatly increased and are apparently sufficient to phosphorylate Rb. Together, these data point to a second noncatalytic role of the cyclin D-dependent kinases, *i.e.*, the mitogen-dependent accumulation of cyclin D-dependent kinases sequesters Cip/Kip proteins, thereby facilitating cyclin E-cdk2 activation (reviewed in Ref. 42). This complements the Rb-E2F transcriptional program (see above) and helps make the appearance of cyclin E-cdk2 activity contingent upon accumulation of cyclin D-cdk4/6-Cip/Kip complexes (Fig. 2).

Once cyclin E-cdk2 is activated, it phosphorylates $p27^{Kip1}$. This converts $p27^{Kip1}$ to a form that is recognized by ubiquitin ligases and is targeted for destruction in proteasomes (43–47). Therefore, cyclin E-cdk2 antagonizes the action of its own inhibitor (Fig. 2). It follows that once cyclin E-cdk2 is activated, unbound $p27^{Kip1}$ is rapidly degraded, contributing to the irreversibility of passage through the restriction point. If cells are persistently stimulated with mitogens, cyclin D-dependent kinase activity remains high in subsequent cycles, $p27^{Kip1}$ levels stay low, and virtually all of the $p27^{Kip1}$ can be found in complexes with the cyclin D-cdks. However, when mitogens are withdrawn, cyclin D is rapidly degraded, and previously sequestered Cip/Kip proteins are mobilized to inhibit cyclin E-cdk2, thereby arresting progression usually within a single cycle.

The *Kip1* gene was not initially thought to be a tumor suppressor, because both copies of the gene were not found to be deleted or silenced in tumor cells. Yet, there is now compelling evidence that *Kip1* is haplo-insufficient for tumor suppression, with loss of only one copy of the gene being sufficient to contribute to cancer (48). In retrospect, we might rationalize this finding through the realization that Cip/Kip proteins are essential for the formation of cyclin D-dependent holoenzymes, although, at least experimentally, $p21^{Cip1}$ can functionally replace $p27^{Kip1}$ in this regard. Still, low levels of $p27^{Kip1}$ (which can be associated with monoallelic *Kip1* deletions in tumor cells) combined with high levels of cyclin E are generally indicative of reduced long-term survival in various forms of cancer. This has been well documented in breast cancer, where the levels of $p27^{Kip1}$ and cyclin E in primary tumors have greater prognostic power than other markers (49, 50). It is particularly important in women without apparent lymph node involvement, in whom the choice of therapy critically depends on strongly predictive markers of this type.

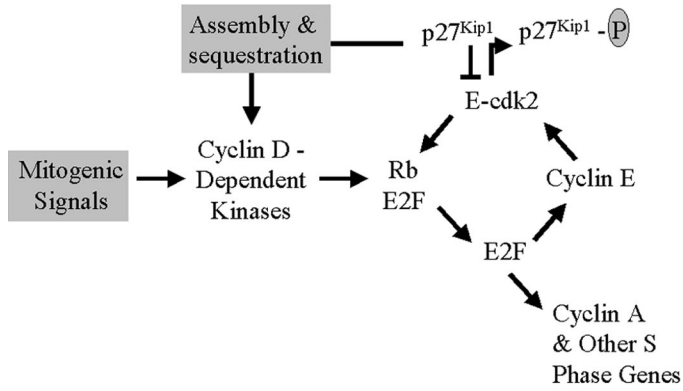

Fig. 2. Restriction point control and the G_1-S transition. As cells enter the division cycle from quiescence, the assembly of cyclin D-dependent kinases in response to mitogenic signals requires Cip/Kip proteins, which are incorporated into catalytically active holoenzyme complexes. The cyclin D-dependent kinases initiate Rb phosphorylation, releasing E2F from negative constraints and facilitating activation of a series of E2F-responsive genes, the products of which are necessary for S-phase entry. Activation of cyclin E by E2F enables formation of the cyclin E-cdk2 complex. This is accelerated by the continued sequestration of Cip/Kip proteins into complexes with assembling cyclin D-cdk complexes. Cyclin E-cdk2 completes the phosphorylation of Rb, further enabling activation of E2F-responsive genes, including cyclin A. Cyclin E-cdk2 also phosphorylates $p27^{Kip1}$, targeting it for ubiquitination and proteasomal degradation. The initiation of the self-reinforcing E2F transcriptional program together with degradation of $p27^{Kip1}$ alleviates mitogen dependency at the restriction point and correlates with the commitment of cells to enter S phase. In subsequent cycles, cyclin D-dependent kinases remain active as long as mitogens are present, and levels of $p27^{Kip1}$ remain low. All $p27^{Kip1}$ in cycling cells is complexed with cyclin D-cdk complexes. Mitogen withdrawal results in cyclin D degradation, liberating $p27^{Kip1}$ from this latent pool. The resulting inhibition of cyclin D- and E-dependent kinases leads to cell cycle arrest, usually within a single cycle.

Cdk Inhibitors: The INK4 Family

Another class of cdk inhibitors, the so-called INK4 proteins (named for their ability to inhibit cdk4), specifically target the cyclin D-dependent kinases (reviewed in Refs. 37 and 51; Fig. 1). INK4 proteins sequester cdk4/6 into binary cdk-INK4 complexes, liberating bound Cip/Kip proteins, and thereby indirectly inhibiting cyclin E-cdk2 to ensure cell cycle arrest (reviewed in Ref. 42). The ability of INK4 proteins to arrest the cell cycle in G_1 phase depends upon the presence of a functional Rb protein, implying that by inhibiting cyclin D-dependent kinases, Rb remains hypophosphorylated and able to repress transcription of S-phase genes (52–54). Note that disruption of cyclin D-cdk complexes and release of bound Cip/Kip proteins is insufficient to inhibit cyclin E-cdk2 in Rb-negative cells. This is likely attributable to the fact that cyclin E-cdk2 activity is normally under Rb-E2F control (Fig. 2), so that cells lacking Rb exhibit greatly elevated cyclin E-cdk2 kinase activity. This enables a conceptually simplified view of the "Rb pathway": INK4 proteins — cyclin D-dependent kinases — Rb — E2Fs → S phase entry.

To date, four INK4 proteins have been identified. These include the founding member $p16^{INK4a}$ (55) and three other closely related genes designated $p15^{INK4b}$ (56), $p18^{INK4c}$ (52, 57), and $p19^{INK4d}$ (57, 58). In humans, *INK4a* and *INK4b* are closely linked on the short arm of chromosome 9 (59), whereas *INK4c* maps to chromosome 1 and *INK4d* maps to chromosome 19. In mice, the *INK4c* and *INK4d* genes are expressed in stereotypic patterns in different tissues during development *in utero*, whereas *INK4a* and *INK4b* expression has not been detected prenatally (60). Gene disruption experiments in mice have revealed no overt effects of *INK4b* or *INK4d* loss (61). In contrast, mice lacking *INK4c* are similar to those lacking *Kip1* in the sense that they have organomegaly and pituitary tumors (62). "Pure" *INK4a*-null mice have not yet been produced (see below). However, one report has provided evidence that inbred BALB/c mice contain defective *INK4a* alleles that encode $p16^{INK4a}$ proteins incapable of inhibiting cyclin D-dependent kinases (63). At face value, the collective data argue that disabling single INK4 family members does not particularly increase the rate of spontaneous tumor development in mice.

Nonetheless, there is compelling evidence that *INK4a* loss-of-function occurs frequently in human cancers (reviewed in Ref. 51). In some familial melanomas, for example, one defective copy of *INK4a* is inherited, whereas the second is lost in tumor cells, the reduction to homozygosity being a classic feature of a tumor suppressor gene (59). In many forms of sporadic cancer, *INK4a* function is also lost (51). For example, virtually all pancreatic carcinomas exhibit *INK4a* defects. As might be expected, the loss of *INK4a* represents only one of several ways in which the Rb pathway can be disabled. In glioblastomas, CDK4 is frequently amplified, and *INK4a* function is lost in other cases. In small cell lung cancer, ~85% of tumors sustain Rb loss, whereas the remaining tumors exhibit *INK4a* loss-of function (10%) or cyclin D amplification (5%; reviewed in Refs. 51 and 64). A remaining puzzle is why other members of the INK4 gene family are not similarly targeted in human tumors. It therefore seems that *INK4a* plays a special role in tumor surveillance in humans. Whatever the reason for the preferential involvement of $p16^{INK4a}$, the available data have led to the reasonable speculation that disruption of the Rb pathway is part of the life history of many, if not all, human tumor cells (reviewed in Ref. 64).

The ARF Tumor Suppressor

Surprisingly, the *INK4a* gene encodes a second potent tumor suppressor (65, 66). The sequences encoding $p16^{INK4a}$ are embodied in three exons (designated 1α, 2, and 3), which specify an mRNA transcript of ~1 kb. In the human and mouse genomes, an alternative first exon (designated 1β) lies 15–20 kb upstream of the $p16^{INK4a}$ coding sequences, and its RNA is spliced to the exon 2 and exon 3 RNA segments to yield a second ~1 kb "β mRNA" whose 5′ end differs from the α transcript (65, 67–69). Alternative promoters located 5′ of exons 1α and 1β govern the independent production of the two mRNAs. The unusual feature is that the initiation codons within exons 1α and 1β are in different reading frames and, when spliced to the same sequences in exon 2, encode two distinct proteins that bear no relationship to one another (65). In the mouse, the ARF protein is represented by 64 amino acids encoded by exon 1β and 105 amino acids specified by exon 2. Mouse $p19^{ARF}$ is a highly basic protein that, when overexpressed, can cause cell cycle arrest in both the G_1 and G_2 phases of the cell cycle (65). Its human counterpart ($p14^{ARF}$) contains fewer exon 2-coded amino acids and is of lower molecular mass, but it has the same ability to induce cell cycle arrest.

Mice containing disrupted *INK4a*/*ARF* exon 2 sequences (70) or lacking only the *ARF* exon 1β sequences (66) are highly tumor prone and die of cancers within 15 months of age. The most predominant tumors are sarcomas, followed by lymphomas, carcinomas, and tumors of the central nervous system (71). *ARF*+/− heterozygotes develop tumors after a considerably longer latency, and the tumor cells lose the wild-type *ARF* allele, as is characteristic of a classical tumor suppressor gene. When MEFs of *INK4a*/*ARF* or *ARF*-null animals are explanted into culture and passaged on a defined 3T3 protocol, the cells do not senesce but rather continue to proliferate as established cell lines (66, 70). Normally, primary MEFs are generally resistant to transformation by oncogenic *Ras* and require the introduction of a so-called immortalizing oncogene, such as adenovirus *E1A* or *Myc*, to undergo transformation (72, 73). However, like established rodent fibroblast lines, *ARF*-null cells can be transformed by oncogenic *Ras* alone (66, 70). In these respects, *ARF*-null MEFs are similar to *p53*-deficient mouse fibroblasts, which are also immortal and can be transformed by Ras without a requirement for Myc or E1A. Moreover, spontaneously immortalized cells derived from a 3T3 protocol contain either mutations in the *p53* gene (80%) or exhibit bi-allelic *ARF* loss (the remaining 20%; Refs. 66 and 74). Together, these data suggested that ARF and p53 functioned in the same biochemical pathway.

p53 is a homotetrameric transcription factor that induces either cell cycle arrest or apoptosis, depending on the biological setting (reviewed in Refs. 75 and 76). Introduction of *ARF* into cells results in p53-dependent cell cycle arrest, indicating that ARF acts "upstream" of p53 (66). Cells lacking p53 alone are refractory to ARF-induced arrest, and in this setting, ARF protein expression is greatly increased. This suggests that p53 suppresses *ARF* expression through negative feedback, and consistent with this interpretation, reintroduction of p53 into these cells returns ARF protein expression to lower levels (Refs. 77 and 78; Fig. 3). ARF stabilizes p53 by antagonizing the p53-negative regulator Mdm2 (77–80). Mdm2 binds to the transactivation domain of the p53 tetramer to inhibit p53-dependent gene expression (81, 82), and it also manifests a ubiquitin ligase activity that appears to target p53 for proteasomal degradation (83). Intriguingly, Mdm2 is itself a p53-responsive gene that normally acts in feedback control to terminate the p53 response (Refs. 84 and 85; Fig. 3). ARF can interfere with all of the known functions of Mdm2, including its ability to: (*a*) block p53 transcription (77–79); (*b*) to ubiquinate p53 (86); and (*c*) to enforce p53 transport into the cytoplasm (87–89), where it is degraded in proteasomes (90–92).

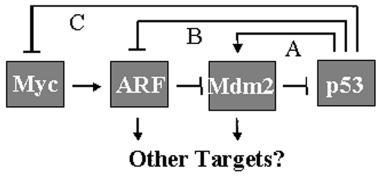

Fig. 3. The ARF-Mdm2-p53 pathway. In response to proteins such as Myc and E2F1 (not shown), ARF protein accumulates and antagonizes the activity of Mdm2. The resulting accumulation of p53 leads to cell cycle arrest or apoptosis, depending on the biological setting. Mdm2 is a p53-responsive gene (pathway *A*) whose p53-dependent accumulation helps to terminate the p53 response. In addition, p53 negatively regulates both ARF (pathway *B*) and Myc (pathway *C*) through as yet unknown mechanisms. ARF likely interacts with targets other than Mdm2, whereas Mdm2 may also functionally interact with proteins other than p53. [This figure is adapted from Eischen *et al.* (100) and is reprinted with permission from *Genes & Development.*]

ARF Connects Rb and p53

ARF expression is activated by abnormal mitogenic signals induced by overexpression of oncoproteins such as Myc (93), E1A (94), E2F1 (95), Ras (96), and v-Abl (97). In this manner, ARF serves to connect the Rb pathway with Mdm2 and p53 (Fig. 4). ARF acts as a fuse to "gate" inappropriate mitogenic signals flowing through the cyclin D-cdk –Rb –E2F circuit, inducing p53 under conditions in which abnormal proliferative signals are generated. This mode of cell-autonomous tumor surveillance diverts cells that have received an oncogenic insult to undergo p53-dependent growth arrest or apoptosis, thereby preventing incipient cancer cells from emerging as overt tumors (reviewed in Ref. 98).

But, if genes like Myc and E1A can induce *ARF* and p53 to trigger growth arrest or cell death, how can these same genes immortalize normal cells and collaborate with oncogenic Ras to transform them? A reasonable hypothesis is that Myc and E1A overexpression, by inducing both cell proliferation and compensating p53-dependent apoptosis, selects for resistant cells that have sustained mutations in the ARF-Mdm2-p53 pathway and that can now be transformed by oncogenic *Ras* alone. To test this idea, primary MEFs were infected with a high titer *Myc* retrovirus, and Myc-induced apoptosis was enforced by depriving the infected cells of serum-containing survival factors. Rare surviving cells were then recloned, expanded as colonies, and genotyped for *p53* mutations and/or *ARF* loss. Strikingly, all such colonies lost the function of p53 or ARF but not both (93). In short, ARF normally acts to protect cells from Myc overexpression by facilitating Myc-induced, p53-dependent apoptosis. Cells corrupted in the ARF-Mdm2-p53 pathway are resistant to Myc-induced killing, enabling Myc to act as a pure growth promoter in this setting.

Studies using animal models support the view that ARF protects cells against Myc-induced tumorigenesis. Mice bearing an Eμ-*Myc* transgene, in which Myc expression is driven by an immunoglobulin heavy chain enhancer, develop Burkitt-type B cell lymphomas with a mean latency of ~30 weeks, and all die of the disease by 1 year of age (99). In the early stages before overt tumors arise, the B-cell compartment of these animals exhibits hyperproliferation, which is balanced by increased apoptosis. When tumors arise, however, the apoptotic index is greatly diminished (100, 101). About 75% of these lymphomas had lesions (*p53* or *ARF* loss, or Mdm2 overexpression) that disabled the ARF-Mdm2-p53 pathway. In addition, when Eμ-*Myc* mice were crossed onto an *ARF*+/− background, tumor progression was greatly accelerated (mean latency, 12 weeks) and 80% of the resulting tumors had lost the wild-type *ARF* allele. Even more strikingly, on an *ARF*-null (100) or *INK4a-ARF*-null (101, 102) background, Eμ-*Myc* transgenic animals all died of highly aggressive lympholeukemias by only 5–6 weeks of age. By contrast, Rb loss of function did not significantly accelerate Eμ-*Myc*-induced disease (101). Most tumors that sustain *ARF* or *p53* mutations do not respond to therapies that can cure mice in earlier stages of lymphoma development (101). Therefore loss of the ability of ARF to modulate the p53 response connotes a poor prognosis, even in those tumors retain wild-type *p53*.

These data imply that by dynamically resetting the effective Mdm2 threshold, ARF reduces the ability of p53 to function in tumor suppression. Consistent with this view, loss of *ARF* makes cells relatively resistant to apoptosis induced by ionizing radiation or cyclophosphamide (94) and can sensitize cells to polyploidy induced by microtubule inhibitors (103). *ARF* loss, like *p53* mutation, can also rescue cells lacking the *Atm* gene from undergoing premature senescence in culture (104), indicating that *ARF* loss modulates the Atm-dependent DNA damage checkpoint. Thus, although DNA damage signals do not appear to activate *ARF per se* (Fig. 4), ARF loss modulates p53 function in such a way as to diminish its accumulation in response to genotoxic stress.

Although the most parsimonious interpretation is that ARF functions in a linear pathway by harnessing the ability of Mdm2 to neutralize p53, there are several reasons to believe that the ARF-Mdm2-p53 pathway has alternative branch points. One line of argument concerns the feedback loops, in which p53 can both induce Mdm2 and repress ARF levels (Fig. 3). The biochemical basis for these connections remains unclear. Moreover, in some *Myc*-induced lymphomas, perturbations were observed that affected expression of more than one gene in the pathway (100). For example, a significant

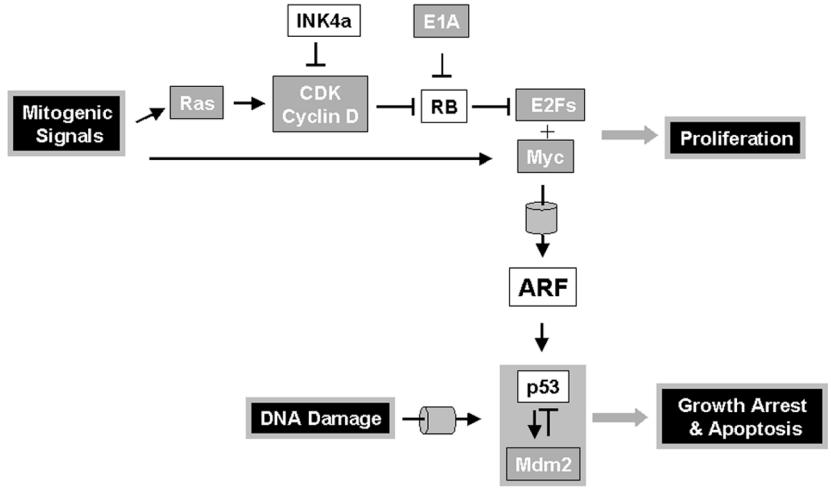

Fig. 4. ARF tumor surveillance. When induced by inappropriate mitogenic signals, ARF antagonizes Mdm2 to activate p53. Hence, hyperproliferative signals are countered by ARF-dependent p53 induction, which diverts incipient cancer cells to undergo growth arrest and/or apoptosis. Loss of the ARF checkpoint (indicated by the *vertical barrel*) subverts this form of cell-autonomous tumor surveillance and allows proteins such as Ras, Myc, E1A, and E2F to function as "pure" proliferation enhancers. DNA damage signals engage various ARF-independent signaling pathways (shown collectively by the *horizontal barrel*) that stabilize p53, most commonly by inducing posttranslational modifications in p53 and/or Mdm2 that prevent their interaction. Although ARF is not directly activated by ionizing radiation or various genotoxic drugs, it is still a potent modifier of the DNA damage response. ARF induction sensitizes cells to DNA damage signals; conversely, ARF loss increases the Mdm2 response and severely dampens the p53 response. All proteins enclosed by *shaded boxes* are potential oncogenes, whereas those illustrated by *unfilled boxes* are tumor suppressors. [This figure is adapted from Sherr (98) and is reprinted with permission from *Genes & Development.*]

fraction of lymphomas exhibited both *ARF* loss and Mdm2 overexpression, implying that both genes can contribute independently to tumor formation. One possibility is that Mdm2 encodes different truncated isoforms, whose as yet undetermined functions may differ from the full-length molecule. At least in principle, ARF might act on targets other than Mdm2, and Mdm2 in turn might regulate proteins other than p53 (Fig. 3). Indeed, there are precedents for the latter, based on reported interactions of Mdm2 with other p53 family members (105), Rb (106, 107), p300 (108), and even E2F1 (109). Much more work is required to critically evaluate these possibilities. Still, it seems evident that disruption of the ARF-Mdm2-p53 pathway occurs frequently in cancers. In humans, *p53* is itself mutated in >50% of cancers, whereas *ARF* loss and Mdm2 overexpression occur in a high fraction of the remaining cases. Hence, disruption of ARF, Mdm2, and p53, like mutations in the $p16^{INK4a}$-cyclin D/cdk4-Rb pathway, again seem to be part of the life history of cancer cells, irrespective of patient age or tumor type.

ARF: In Search of Biochemical Function

ARF is a highly basic protein that localizes to the nucleolus (65, 79, 89). When induced or overexpressed, ARF binds to Mdm2 and imports it into the nucleolus, thereby allowing *p53* to accumulate in the nucleoplasm (89, 110, 111). Recently, ARF was found to bind to a central region of Mdm2 to a segment distinct from Mdm2's nuclear import and export signals, its NH_2-terminal *p53* binding domain, and the COOH-terminal RING domain, the integrity of which is required for E3 ubiquitin ligase activity (111). Both the human and mouse ARF proteins contact Mdm2 through two independent binding sites that are separated by spacer elements of different lengths in the two proteins. In mouse $p19^{ARF}$, the two Mdm2 binding sites cluster in the ARF NH_2 terminus within amino acids 1–37 (111). Segments containing amino acids 1–14 and 26–37 are responsible for cooperative binding and induce an allosteric change in Mdm2 that facilitates its nucleolar import. Interestingly, a cryptic localization signal within the COOH-terminal Mdm2 RING domain contributes to the nuclear import of the ARF-Mdm2 complex. Mutations within this region prevent Mdm2 nucleolar import and instead result in ARF sequestration by Mdm2 in the nucleoplasm (110, 111). The fact that Mdm2 RING domain mutants can oppose the activity of ARF implies that the ARF-Mdm2 interaction is bidirectional, with each protein having a potential to cancel activities of the other.

Although the spacing between the Mdm2 binding domains in the human $p14^{ARF}$ protein is greater than that in mouse $p19^{ARF}$ (88), human or mouse ARF mutants that either do not interact properly with Mdm2 or colocalize Mdm2 to the nucleolus are impaired in arresting cell proliferation (89, 110, 111). To date, these functional data suggest that the ability of ARF to sequester Mdm2 correlates with *p53*-dependent cell cycle arrest. However, it is formally possible that ARF might also antagonize Mdm2 in the nucleoplasm (88). These findings raise interesting questions about the *in vivo* activities of ARF. Is the primary role of ARF to sequester Mdm2 from p53 (89, 110, 111), to interfere with Mdm2-catalyzed ubiquitination (83), to prevent Mdm2 from enforcing p53 nuclear export (87–89, 110, 111), or all of the above?

Conclusions and Future Prospects

In summary, studies over the last decade have indicated that most human cancer cells sustain mutations that affect the functions of Rb and *p53*, either by disabling these genes directly or by targeting genes that act epistatically to prevent their proper function. The *INK4a*/*ARF* locus surprisingly encodes two products that affect both Rb and *p53*, and the rationale for nature's design of these overlapping tumor suppressors continues to pose a puzzle. An implication may be that the activities of *INK4a* and *ARF* are somehow coregulated through their proximity in the genome, although much of the data collected thus far argue against this interpretation. There is clearly more to learn here.

If, in fact, it is true that disabling the Rb and p53 pathways is a hallmark of cancer, then the most efficacious treatment would be to restore their functions. The inability to specifically target genes to tumor cells and to properly regulate their expression makes "gene therapy" impractical. Novel therapeutics will likely need to target ancillary pathways. Can we take advantage of weaknesses in tumors lacking Rb and/or p53 to selectively kill them? One rationale is based on the concept of "synthetic lethality" in yeast, in which disruption of one gene–in this case, Rb and/or p53–might sensitize cells to disruption of another pathway while sparing cells that retain either one of the two functions. Cyclin A-cdk2 activity is required to terminate E2F function in S phase, and blocking this function triggers apoptosis (112, 113). One idea, then, is that cells that lack Rb and *p53* checkpoints might prove more sensitive than normal cells to cdk inhibitors (114), which are now being widely developed. The recent realization that cells lacking a p53-inducible nuclear subunit of ribonucleotide reductase may rely on a cytoplasmic form of this enzyme to resist drug-induced genotoxic damage (115) may provide another opportunity for targeted therapy. Others have speculated that if specific inhibitors of the cytoplasmic form of ribonucleotide reductase could be developed, these might selectively sensitize cells with mutant p53 to DNA-damaging chemotherapeutic agents (116). Yet another approach would be to activate the apoptotic machinery downstream of the sensory signals that normally lead to *p53*-dependent activation, *e.g.,* by activating death-inducing receptors that couple to caspases (117). An article of faith is that a better understanding of cancer cells will lead to new drug targets and novel therapeutic approaches–that good science will lead to good medicine. Here, again, I quote from Thomas:

"It is much more difficult to be convincing about ignorance concerning disease mechanisms than it is to make claims for full comprehension, especially when the comprehension leads, logically or not, to some sort of action. When it comes to serious illness, the public tends, understandably, to be more skeptical about the skeptics, more willing to believe the true believers. It is medicine's oldest dilemma, not to be settled by candor . . . What it needs is a lot of time and patience, waiting for the science to come in, as it has in the past, with the solid facts" (118).

We should be optimistic that the learning curve is accelerating.

Acknowledgments

I thank the Pezcoller Foundation and the AACR for honoring me with this prize. All scientists build their careers on the shoulders of those who taught them and the many who have worked with them, both as colleagues and trainees. I thank my mentors, Ross Basch, Jonathan Uhr, Burton Goldberg, and George Todaro, for their guidance early in my career; my many colleagues at the NIH and St. Jude Children's Research Hospital over a period of >25 years; and in particular, the many postdoctoral fellows who have shared in discovery along the way. Finally, I thank my wife, Martine Roussel, who, apart from everything else in our lives together, has worked closely with me in the laboratory for the last 17 years–as a Leo, she deserves a lion's share.

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