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Review Article

p16^{INK4a} as a tumor suppressor with therapeutic applicability

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CONTENTS

Abstract

The *p16*$^{INK4a/ARF}$ locus encodes for 2 tumor suppressor genes commonly lost during transformation and acquisition of malignancy processes. The product of *p16*^{INK4a} gene is a CDK inhibitor that constrains cell cycle progression in response to different proliferative stresses and participates in cell-environment signaling pathways. Restitution of *p16*^{INK4a} function has showed potent antitumoral activity in new gene therapy approaches and should be taken into consideration for future design of rationale and specific therapeutic strategies against cancer.

p16 as a tumor suppressor gene

A complicated network of regulatory activities in the cells of pluricellular organisms limits their proliferation to strictly necessary situations (*i.e.*, tissue production and regeneration, growth) and guarantees the quality of the genetic material inherited by daughter cells. In this network, positive regulators may act as oncogenes or oncoproteins when activated aberrantly. On the other hand, loss of negative regulators may result in uncontrolled proliferation. Tumor suppressor genes are those that have antiproliferative activity and their loss causes sensitivity to deregulated growth and neoplastic transformation.

Several tumor suppressor genes participate in senescence and apoptosis pathways avoiding aberrant proliferation in response to oncogenic stress (*i.e.*, misexpression of oncogenes, DNA instability, DNA damage, *etc.*). Senescence represents a limit in the life span of proliferating cells and results in irreversible cell cycle arrest. Apoptosis leads to the elimination of potentially dangerous cells by programmed cell death.

At least 3 different but related senescence-inducing pathways have been described (1): the p16^{INK4a}/pRb pathway, the ARF/p53/p21^{Cip1} pathway and the PTEN/p27^{Kip1} pathway. Inactivation of some or all of these pathways seems to be necessary for prolonged life span and unlimited proliferation.

The *p16*$^{INK4a/ARF}$ locus encodes for 2 important gene products that participate directly in senescence and apoptosis pathways. This locus has been mapped to *9p21*, a region often found to be rearranged in a large proportion of tumors and cell lines (2). Primary tumor samples from melanomas, adenocarcinomas and glioblastomas contain homozygous deletions of this gene (3-7). Furthermore, linkage analysis has demonstrated that 9p21 is one of the loci involved in predisposition to familial melanoma (8) and hereditary pancreatic carcinoma (9). The putative tumor suppressor gene localized in 9p21 was initially called *MLM* (10) or *MST-1* (Multi-Tumor Suppressor 1) (2).

The first gene in 9p21

The first product from this locus to be described was p16^{INK4a}, a cyclin-dependent kinase (CDK) inhibitor (CKI) that binds to the monomeric forms of CDK4 and CDK6 and impedes the formation of active kinase complexes with the regulatory subunit (cyclin D1, D2 or D3) (11). CDK4/6-CycD complexes are part of the kinase

activities that regulate the G_1-to-S phase transition, and represent the sensor that communicate the mitogenic signals (*e.g.*, growth factors, oncogenic stress) to the cell cycle machinery (see below). Furthermore, the negative regulation of these activities integrates several intracellular and extracellular antiproliferative signals (*i.e.*, growth-inhibiting factors, contact inhibition, ageing, cellular stress, *etc.*).

The role of $p16^{INK4a}$ in a senescence pathway was described in a model of primary human and murine proliferating fibroblasts transfected with *H-Ras*. Expression of oncogenic *Ras* in primary cells resulted in permanent G_1 arrest accompanied by accumulation of p53 and p16 (12). In the absence of *p16* and/or *p53*, *Ras*-induced senescence did not proceed and oncogenic transformation occurred. In addition, in cells lacking $p16^{INK4a/ARF}$ gene, the overexpression of $p16^{INK4a}$ was sufficient to avoid transformation by oncogenic *H-Ras* (13). p16 protein also accumulates in response to activation of Raf (14) and MEK/MAPK (15), cell-doubling accumulation (16-18) and DNA damage (19). In summary, all these data suggest that $p16^{INK4a}$ has a role in controlling cell life span and avoiding transformation by oncogenic stresses.

Furthermore, the product of the retinoblastoma predisposing gene (*pRb*) is one of the targets of CDK-cyclin activity, and is thought to be one of the main effectors of p16 antiproliferative signaling due to its function as an E2F-dependent transcription silencer (20, 21). Interestingly, $p16^{INK4a}$ and *pRb* show a reciprocal pattern of alterations in transformed cells. Only few tumors retain both $p16^{INK4a}$ and *pRb*, but *CDK4* or *CycD1* overexpression are often found in these cases (reviewed in 22, 23). In a particular but interesting case, a mutation in *CDK4* (R24C) that abrogates p16 binding was also found in melanoma-prone families (24, 25). In conclusion, the $p16^{INK4a}$/CDK4/CycD1/pRb pathway is deregulated in most human tumors either by loss of the tumor suppressors $p16^{INK4a}$ or *pRb*, or by activation of the oncogenes *CDK4* or *CycD1* (26, 27).

In spite of intense efforts to understand $p16^{INK4a}$ function, little is known about regulation and expression of this gene. In addition to the well-characterized upregulation of *p16* expression in the above mentioned presenescent situations, some reports suggest a negative feedback with *pRb* during the cell cycle (17). It has been proposed that active pRb could repress *p16* expression (28) and that inactivation of *pRb* would result in increased p16 levels and block CDK4/6-CycD activity. However, no obvious E2F sites were found in the $p16^{INK4a}$ promoter, suggesting that the effects may be indirect. Recent work has defined JunB (AP-1) as a transcription factor that upregulates $p16^{INK4a}$ expression, linking mitogenic signaling to $p16^{INK4a}$ upregulation (29). Two conventional consensus binding sites for the Sp-transcription factor family of proteins are contained in a region necessary for senescence-induced *p16* overexpression (30). The same study also defined a silencer region in the promoter of $p16^{INK4a}$, which may be occupied by a putative transcription inhibition factor that is highly expressed in young cells. Negative regulators Bmi-1 (31) and Id1 (32, 33) have been reported to repress $p16^{INK4a}$ expression at the transcriptional level.

An overlapping gene in 9p21

One of the most surprising discoveries of recent years was the description of an overlapping gene in the $p16^{INK4a}$ locus that encodes a completely unrelated protein with antiproliferative and putative tumor suppressive activity. In July 1995, 3 different groups reported an alternative mRNA sharing exons 2 and 3 with the known $p16^{INK4a}$ transcript (34-36). Surprisingly, each gene had its own exon 1 and the ORFs predicted were not coincident. Some months later Sherr's group (37) described, in murine cells, p19ARF (for Alternative Reading Frame) as the product of the alternative transcript of the $p16^{INK4a}$ locus, which had no amino acid sequence homology with p16 protein (Fig. 1). The human homologue for p19ARF was called p14ARF (since it was smaller). Mouse p19ARF and human p14ARF are nuclear proteins that induce G_1 and G_2 phase arrest when introduced into a variety of cell types (reviewed in 38; more data in 37, 39) in a p53-dependent manner (40). ARF protein stabilizes and activates p53, resulting in upregulation of p53 target genes, such as $p21^{Cip1}$, *Bax* or *MDM2*, and inducing cell cycle arrest or apoptosis. ARF proteins seem to interfere in the p53-degradation pathway by binding to and inactivating MDM2 (HDM2 in humans), the main ubiquitin ligase E3-type enzyme for p53 (41). In addition, it has been reported that murine cells lacking both p53 and MDM2 are surprisingly sensitive to p19ARF-induced arrest, indicating that ARF is able to induce cell cycle arrest through targets other than p53 (42).

Similar to $p16^{INK4a}$, *ARF* is overexpressed in response to oncogenic stress and when cells progress towards senescence (40, 43, 44) and loss of ARF activity results in immortalization and sensitivity to oncogenic transformation by *Ras*, at least in murine primary fibroblasts. Furthermore, the ARF/MDM2/p53/p21Cip1 pathway is deregulated in most human tumors either by loss of tumor suppressors $p19\text{-}p14^{ARF}$ or *p53*, or by activation of the oncogene *MDM2* (reviewed in 38, 45, 46).

The expression of *ARF* is negatively controlled by p53-transrepressing activity, closing a regulatory loop (ARF-MDM2-p53) that allows for rapid, strong control of p53 function. Interestingly, *ARF* is also a transcriptional target of E2F-1, linking the proliferation signal to the activation of senescence and apoptotic pathways (reviewed in 47). Loss of pRb function or increases in E2F-1 activity not only results in expression of the genes necessary for proliferation (*CycE*, *CycA*, *Cdc2*), but also in induction of senescence and apoptosis pathways that may restrain aberrant proliferation through the expression of *ARF* and other different apoptotic effectors (*i.e.*, caspases, Apaf-1, p73).

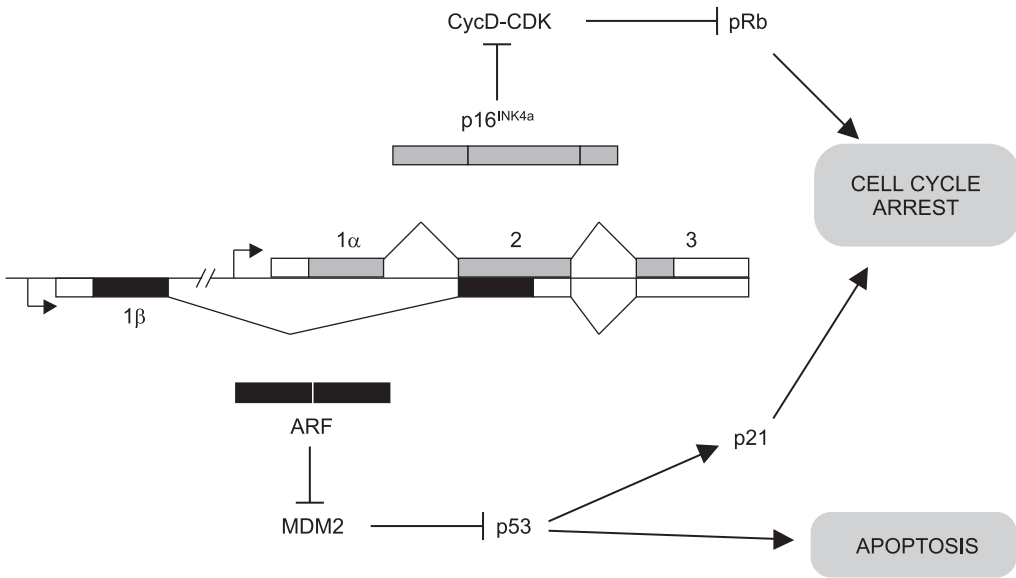

Fig. 1. Organization of the *p16^{INK4a}*/ARF locus. Two genes are partially overlapped in chromosome *9p21*. Exons are indicated by boxes. Grey and black boxes are translated regions encoding for p16^{INK4a} and ARF proteins, respectively. Each gene product participates in the 2 main regulatory pathways that control cell proliferation and apoptosis. (Adapted from 37, 45).

p16^{INK4a}/ARF genes inactivation

Although initial reports found frequent deletions and point mutations in the p*16$^{INK4a/ARF}$* locus in primary tumors and established cell lines, it remained unclear whether the inactivation of the 2 genes was a frequently coordinated event or rather each gene had independent inactivation mechanisms. Did the status of 1 gene depend on the integrity of the other? The *p16^{INK4a}* gene has been found to be inactivated by homozygous deletions, point mutations and *de novo* promoter methylation (reviewed in 22). Extensive deletions in *9p21* usually affect both genes, together with the nearby *p15^{INK4b}* gene. However, other frequent small deletions do not necessarily target both genes. Moreover, point mutations that affect the *p16^{INK4a}* gene rarely affect *ARF* functionality. *p16^{INK4a}*-inactivating sequence variants are distributed throughout the entire coding region, but only those corresponding to exon 2 may have an effect on ARF protein. Surprisingly, in the murine system and in human glioma cell lines hardly any of the aberrations in the exon 2 shared region affect the ability of ARF to cause cell cycle arrest (48, 49). No point mutations in exon 1beta were found in screenings of primary tumors and established cell lines, and very few cases of specific *p14^{ARF}* deletions leave the *p16^{INK4a}* coding region intact (reviewed in 22, 23, 50). *De novo* promoter methylation is an important mechanism of gene silencing that targets individual genes (51). *p16^{INK4a}* promoter methylation is a very frequent event in primary tumors and established cell lines (52), especially in primary myeloma, bladder, colon, esophagus and breast cancer (20-75%; reviewed in 22). Frequent *p14^{ARF}* promoter methylation has also been described in several primary tumors (53). Silencing of each promoter is independent of the methylation status of the other (54), representing a significant event for individual *ARF* inactivation. Taken together, the extensive data reported in the literature suggest that genetic aberrations preferentially target *p16^{INK4a}* although they may result in consequent *ARF* losses, while promoter methylation independently targets *p16^{INK4a}* or *ARF* genes (23, 50).

However this interpretation is not unique and must be taken carefully. Future studies must consider genetic and epigenetic changes affecting all the genes involved in senescence and apoptosis regulatory pathways, rather than single or paired targets. Moreover, new screenings should observe likely tissue specificities, the chronology of events and the partial but unavoidable dependence of the "two-genes-one-locus" genes.

Two-genes-one-locus

All the data mentioned above render a very uncommon scenario: 2 important negative regulators of cell proliferation overlap in a unique locus that is frequently lost or inactivated in many primary tumors. This idea raised important questions, some of which are still unanswered.

The first question was: are both *p16^{INK4a}* and *ARF* tumor suppressor genes or is one of them dispensable for limiting oncogenic transformation? To address this issue, great efforts have been made to reproduce the loss of each gene in a murine model. Serrano *et al*., constructing a knockout (KO) of exons 2 and 3 (*INK4a/ARFδ2,3*), published the first attempt in 1996 (55). Ablation of the shared

coding region inactivated both genes. *INK4a/ARF*-deficient mice were viable, but developed spontaneous tumors (sarcomas and lymphomas) at an early age with a lethality of 90% in the first 12 months, and were highly sensitive to carcinogenic treatments. In addition, MEFs from these mice were resistant to Ras-induced senescence, grew rapidly and had high colony-formation efficiency (55). The specific disruption of exon 1β rendered a $p19^{ARF}$-deficient mouse that almost completely reproduced the *INK4a/ARFδ2,3* phenotype (40), challenging the role of $p16^{INK4a}$ as a murine tumor suppressor gene.

This debate was open until recent publications of "pure" $p16^{INK4a}$ KO mice by 2 different groups (56, 57; see commentary by Sherr in 58). In spite of the differences in methodology used and results obtained, both groups reported a weak phenotype for the pure $p16^{INK4a}$ KO: mice developed normally and only a small incidence of spontaneous tumors was detected during the first year of life. However, all agreed that $p16^{INK4a}$ is a tumor suppressor because $p16^{INK4a}$ null mice are more sensitive to carcinogens and because loss of *INK4a* can cooperate with *ARF* heterozygosity to yield a wide spectrum of tumors (resembling the situation in the familial melanoma syndrome). In addition, although both groups agree that loss of $p16^{INK4a}$ alone is insufficient for immortalization of MEFs, its absence may facilitate their escape from senescence. However, similar experiments using murine primary cells from different origins (pre-B cells and macrophages) (59) or human primary cells (60) suggest a more important role of $p16^{INK4a}$ in immortalization and tumor suppression. For instance, ^{INK4a}-deficient human diploid fibroblasts are resistant to Ras-induced senescence (60) in contrast to the $p16^{INK4a}$-KO MEFs.

Moreover, there are 2 additional clues that indicate that $p16^{INK4a}$ is a real tumor suppressor: first, specific genetic (intragenic mutations and microdeletions) or epigenetic (promoter methylation) inactivation of $p16^{INK4a}$ with retention of at least *ARF* in heterozygosity is frequent in primary tumors; and second, loss of $p16^{INK4a}$ compensates for retention of pRb activity and vice versa as necessary events in tumorigenesis.

The second, unsolved question remains: why do these 2 important tumor suppressor genes overlap structurally? At first glance, it would appear that the presence of 2 overlapping genes, both of which are involved in the 2 most important pathways that protect from aberrant proliferation, constitutes a high risk. In addition to the intimate relationship at the genetic level, $p16^{INK4a}$ and ARF participate in 2 highly imbricate pathways conforming a signaling network that is central to tumor formation. Therefore, there must be some evolutionary advantage implied by this unprecedented situation. Coregulation of both genes has been proposed as explanation, but it seems unlikely because $p16^{INK4a}$ and *ARF* are only coexpressed when primary cells are explanted into culture and progress towards senescence. On the other hand, cells possess other, less dangerous mechanisms to coordinate expression of genes.

Biological activities of $p16^{INK4a}$

p16 as a cell cycle regulator

As already mentioned, $p16^{INK4a}$ was first described as a protein that specifically binds and inhibits CDK4 and CDK6, but not CDK2. Three other, related proteins ($p15^{INK4b}$, $p19^{INK4d}$ and $p18^{INK4c}$) were described soon after, sharing structural and functional properties and conforming to the INK4 family of CKI. However, none of these 3 new members of the family are directly related to tumor suppression (with the exception of $p15^{INK4b}$, localized close to $p16^{INK4a}$ on chromosome 9p21 and commonly codeleted). Another family of CKI, the Cip/Kip family had been characterized. The components of this family ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$) bind CycD-CDK4/6 and CycE/A-CDK2 complexes, and were initially understood to inhibit all these complexes. CDK inhibition by any of these CKIs was originally thought to result in hypophosphorylation of pRb and E2F transcriptional activity silencing, causing arrest in the first gap (G1) phase of the cell cycle (reviewed in 61).

However, compilation of data from the last few years has outlined a much more complicated scenario in which CycD-CDK4/6-INK4 is a sensor that integrates several endogenous and exogenous signals to finally turn on or turn off the autonomous machinery of progression through the cell cycle. Next, we briefly address the description of the G_1-to-S transition switch (classically named "restriction point") and the requirements for p16-mediated cell cycle arrest.

Complex regulation of CDK

Progression through the G_1 phase of the cell cycle is mainly controlled by phosphorylation of substrates by the cooperative and sequential action of G_1-CDK (basically, CDK4/6 and CDK2). To minimize the risk of aberrant proliferation, CDK activities are complexly regulated by: a) protein levels of the regulatory subunit (cyclins); b) assembly of complexes; c) phosphorylation of Cyclin-CDK complexes by CDK-activating kinase (CAK); d) localization of complexes; e) binding to inhibitory domains (CKI). In turn, G_1/S cyclins are usually very short-lived, and their accumulation is tightly controlled at transcription and at degradation levels in a cell cycle phase-dependent manner. Members of INK4 family of CKI are quite stable proteins and their accumulation depends basically on transcription control. In contrast, turnover of Cip/Kip proteins seems to play a major role in the overall control of CDK activity. Surprisingly, recent data have demonstrated that Cip/Kip proteins act as positive regulators of CDK4/6 activity, participating in CycD-stabilization, CycD-CDK4/6 complex assembly, and directing active complexes to the nucleus (62). Nevertheless, when Cip/Kip-CycD-CDK4/6 complexes are disrupted by INK4 binding or CycD1 downregulation, Cip/Kip proteins binds to and strongly inhibits CycE/A-CDK2 activity (reviewed in 61).

pRb family: "pocket" proteins

The *Rb* gene was originally identified as a tumor suppressor gene linked to the hereditary predisposition to retinoblastoma. Sporadic mutations or inactivation of the pRb pathway are common in human tumors. Several viral oncoproteins were found to bind and inactivate pRb, showing the relevance of pRb function in the control of cell cycle progression. However, these viral oncoproteins bind equally to 2 other proteins, p107 and p130, that share structural and functional properties with pRb (63, 64). All 3 proteins have a large homology region (pocket) responsible for binding to members of the E2F family of transcription factors. Pocket proteins are major regulators of cell progression through their ability to modulate E2F transcriptional activity (reviewed in 65). At least 6 different E2F transcription factors have been described (reviewed in 66). E2F-1, -2 and -3 are positive transcriptional regulators of genes necessary for S phase entry, and are negatively regulated by binding to hypophosphorylated pRb. E2F-4 and E2F-5 act as transcriptional repressors when bound to any of the pRb family members during G0-G1 phase. E2F-6 is very different from its relatives since it does not bind any pocket protein and lacks transactivation domain. In an extremely simplistic summary, G1 phase Cyc-CDKs phosphorylate and inactivate pocket proteins, rendering activation of E2F-dependent expression and entry into the S phase of the cell cycle. However, we should not forget that pocket proteins are differentially expressed during the cell cycle and phosphorylated differently by complexly regulated Cyc-CDK activities (67, 68). They have particular and overlapping functions and different affinities for each E2F family member (69) and they can play additional and important roles. For instance, p130 is expressed in G0, essentially inactivated by CycE-CDK2 and rapidly degraded during S phase (67, 70), while p107 is expressed in S phase (67) and preferentially phosphorylated by CycD1-CDK4 (71). Both p130 and p107 can bind to CycA/E-CDK2 complexes (72) acting as a CKI (73). pRb is thought to be the only pocket protein that binds to E2F-1 and pRb-E2F1 complexes are disrupted after coordinated phosphorylation by CDK4 and CDK2 (74). So far it is not clear whether CycD1-CDK4 inactivates pRb or helps the formation of pRb-E2F-1 complexes (75).

Mitogenic signal: turning on the G_1-S switch

In response to mitogens (*i.e.,* growth factors), a number of signaling pathways are activated (Fig. 2A) (61). The G_0/G_1 to S phase transition, or the decision to proliferate, can be organized in 3 steps:

a) Activation of CycD1-CDK4/6 complexes: mitogen-induced Ras-Raf-MEK-ERK signaling pathway promotes transcription of *CycD1*, expression of *p21^{Cip1}* and assembly of active complexes. In addition, the Ras-PI3K-Akt-GSK3β pathway cooperates with newly-synthesized p21^{Cip1} to reduce turnover of CycD1.

b) Activation of CycE-CDK2 complexes: Active CycD-CDK can partially phosphorylate pRb and allow some CycE expression. In addition, titration of p21^{Cip1} and p27^{Kip1} into CycD1-CDK4/6 complexes rescues CycE-CDK2 from Cip/Kip constraint. Moreover, mitogen-induced MAPK and PI3K/Akt pathways collaborate with new active CycE-CDK2 complexes to phosphorylate p27^{Kip1} and direct its degradation. As a result, CDK4/6 and CDK2 are fully activated.

c) E2F-1 expression and activation of responsive genes. Cooperative phosphorylation of pRb and p130 disrupts E2F-4/pRb and E2F-4/p130 complexes, resulting in derepression of some E2F-responsive genes (for example E2F-1 and p107). Newly expressed E2F-1 cannot be recruited by hyperphosphorylated pRb and transactivates S phase E2F-1-responsive genes (*e.g.*, CycA, DHFR, *etc.*).

p16^{INK4a} expression: shutting down the cell cycle engine

Expression of *p16^{INK4a}* or any other INK4 family member is an effective brake to cell proliferation due to its ability to coordinately inhibit all G_1-Cyc-CDK activities (Fig. 2B). First, p16^{INK4a} binds to CDK4/6 and disrupts Cip/Kip-CycD1-CDK4/6 active complexes. This results in CycD destabilization and degradation, and release of p21^{Cip} and p27^{Kip1}. Free Cip/Kip proteins then inhibit CycE/A-CDK2 complexes, leading to complete G_1-CDK activity inhibition and pocket protein hypophosphorylation. In this situation, active forms of pRb, p130 and p107 inhibit E2F-1 transcription and form transrepressor complexes with E2F-4. To note, it has been reported that p16^{INK4a}-mediated cell cycle arrest requires both pRb and p130/p107 activities (76). S phase-required genes (such as *CycE*, *CycA*, *Cdc2*, *E2F-1*, *etc.*) are rapidly silenced, and cells remain arrested in G_1 phase, acquiring senescence or terminal differentiation features.

According to this model, CycD1 is the main sensor of mitogenic signals and INK4 is the main antiproliferative brake. However, deregulation of G_1 to S transition machinery in any point downstream of CycD-CDK-INK4 may result in uncontrolled progression through the cell cycle and mitogen-independent, INK4-irresponsive cell proliferation. Indeed, loss of *pRb* is sufficient to overcome *p16^{INK4a}* restrictive function, so tumors harboring *pRb* defects usually retain *p16^{INK4a}* gene and expression (even at high levels) (20, 21). E2F-1 overexpression (77), *CycE* constitutive expression (78, 79) and low p27^{Kip1} protein levels (80) can also lead to external signal-independent growth.

Other biological actions of p16

To date, the only well characterized biochemical activity of p16^{INK4a} is the binding to and inactivation of CDK4/6. However, recent experimental evidence impli-

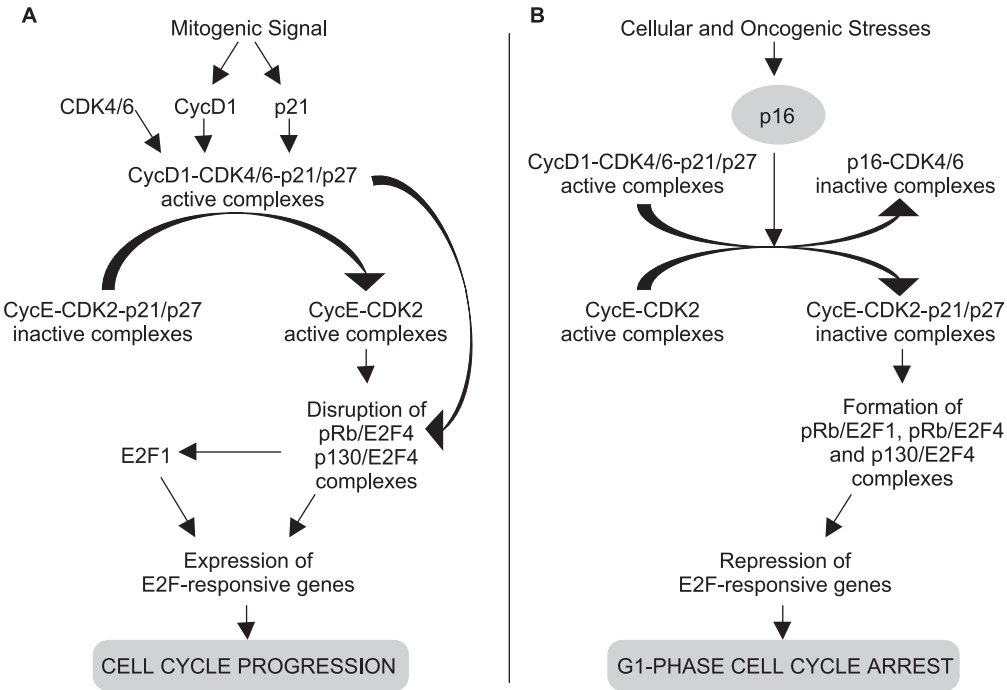

Fig. 2. p16INK4a as a cell cycle regulator. (A) Mitogen-induced cell proliferation is dependent on G_1-CDK activities, phosphorylation of pocket proteins and expression of E2F-dependent genes. (B) p16INK4a exerts its cell cycle blocking action through coordinated inhibition of G_1-CDK activities, pocket proteins hypophosphorylation, and repression of E2F-responsive genes.

cates p16^{INK4a} in the control of important processes other than cell cycle progression constraint. Several groups reported the ability of p16^{INK4a} overexpression to induce apoptosis (see below) either in a p53-dependent or p53-independent manner. Sandig and coworkers reported interesting cooperative effects of p16^{INK4a} and p53 in the induction of apoptosis (81). Furthermore, the participation of p16^{INK4a} in a DNA damage checkpoint has been observed (19, 82).

More surprising is the finding of p16^{INK4a} participation in cell-cell and cell-extracellular matrix (ECM) interaction control. For instance, overexpression of p16^{INK4a} inhibits $\alpha_v\beta_3$ integrin-mediated cell spreading (83) and glioma invasion (84) *in vitro*. There is also evidence of p16^{INK4a}-induced apoptosis upon loss of anchorage (anoikis) in a variety of human cancer cells (85). Moreover, restoration of p16^{INK4a} expression results in vascular endothelial growth factor (VEGF) downregulation and inhibition of angiogenesis (86, 87). In summary, loss of p16^{INK4a} has biological consequences other than aberrant cell proliferation, such as loss of contact inhibition (88) and anchorage-dependence, acquisition of migratory and invasive features and stimulation of angiogenesis, which might cooperate in neoplastic and metastatic processes. However, it is still unclear how these actions are dependent on CDK4/6 inhibition and whether they are a consequence of cell cycle arrest. p16^{INK4a} may have other biochemical roles, although to date there is no evidence of binding or interaction with other targets.

p16 as a therapeutic agent

So far, we have addressed whether *p16^{INK4a}* is a tumor suppressor gene, how it is mutated, deleted or inactivated in human tumors, what its biological activities are and the significance of its loss in terms of tumor formation and metastasis. The data available point to *p16^{INK4a}* gene as a good target for cancer therapy. In this case, the therapeutic strategy must be the restoration of the lost function. At least theoretically, restoration of p16^{INK4a} might stop tumor cell proliferation, invasion and metastasis without affecting healthy tissues. However, from the data reported here we can infer a limit for p16^{INK4a} effectiveness against tumor progression: p16^{INK4a}-induced cell cycle arrest is dependent on a functional p16/CycD1/CDK4-CycE/CDK2-pRb/p130 pathway. In general, tumor cells retaining the wild type (wt) *p16^{INK4a}* gene and protein expression are resistant to p16^{INK4a} activity due to downstream defects (*CycD1* or *CycE* overexpression, loss of *pRb*, constitutive E2F1 activity). The status of *p16^{INK4a}* gene and expression may have a prognostic value, both for the severity of the malignancy and for the predicted outcome after specific therapies.

Nevertheless, *p16^{INK4a}* loss or inactivation is one of the most frequent genetic defects in certain cancers, such as melanoma, pancreatic carcinoma and invasive glioblastoma, and is an important event in many other malignancies (22, 23). In consequence, scientists have

long tried to design rational strategies to reintroduce this lost function into cancer cells. Some of the most promising approaches are discussed below.

Reexpression of endogenous inactivated p16^{INK4a} using demethylating agents

Together with homozygous deletion and point mutation, *de novo* promotor methylation and silencing is an important mechanism of *p16^{INK4a}* inactivation (see above). Moreover, methylation of *p15^{INK4b}* was detected in 82% of acute myeloid leukemia and 57% of acute lymphoblastic leukemia samples(reviewed in 22). Treatment of cell lines with demethylating agents, such as decitabine, has long been used to test promoter methylation and to restore expression of silenced genes. It has also been shown that decitabine can restore the expression of endogenous silenced *p16^{INK4a}* (89). Therapeutic strategies based on reexpression of endogenous *p16^{INK4a}* have been tested on different tumoral cell lines, and several reports agree that decitabine can cause cell cycle arrest and senescence (87, 90, 91), correlating with *p16^{INK4a}* reexpression in most but not all cases. Therefore, it is still unclear whether this activity is dependent on *p16^{INK4a}* reexpression or the drug has pleiotropic effects that define its cytotoxicity. In addition, demethylating agents can cause misregulation of physiologically methylated genes, resulting in unspecific, unexpected effects. Phase III trials involving patients with myeloid leukemia using decitabine show promising clinical activity (92). In contrast, attempts to use decitabine to treat solid tumors failed in most of the phase II trials.

Gene therapy, reintroduction of full-length wt-p16INK4a cDNA

Another exciting possible therapy is the transfer of genetic material. Cancer gene therapy based on restitution of the tumor suppressor gene p53 has long been studied in a number of models *in vitro* and *in vivo* and in several clinical trials. The large experience accumulated on the way to a successful p53-transfer-based cancer therapy may teach us a lot about the possibilities and limitations of these strategies. While results obtained *in vitro* with tumor cell lines showed high transference efficiency and treatment efficacy and were very encouraging, *in vivo* preclinical models showed the greatest challenge: the limited delivery of the therapeutic gene to restricted parts of the tumor. Clinical trials for gene therapy with p53 have shown controversial results. In summary, it is now clear that future research directions will include development of more efficient vectors, use of novel genes and combined modality approaches (reviewed in 93). Given its characteristics, the *p16^{INK4a}* tumor suppressor gene could be one of the next therapeutic agents to be taken into consideration. The *p16^{INK4a}* gene has been transfected in a number of tumor cell lines and animal models in an attempt to understand mechanisms of action and to identify possible therapeutic applications.

Retrovirus-mediated reintroduction of the wt *p16^{INK4a}* gene has been addressed *in vitro* in glioma (94), breast cancer (95), esophageal SCC (90), T-ALL and AML (96-98) cell lines, and *in vivo* in a glioma xenograft model (99) and tumorigenesis assays (96, 100). Results *in vitro* showed the ability of newly expressed *p16^{INK4a}* to inhibit cell proliferation and cause an accumulation of cells in the G1 phase of the cell cycle. Cell cycle arrest was found to be dependent on pRb hypophosphorylation (90, 98). Transformed cells retrovirally transduced and expressing exogenous p*16^{INK4a}* lost their tumorigenicity in a model of murine T-ALL (96) and in a breast cancer nude mice xenograft model (100). Interestingly, treatment of previously established glioma xenografts with retro-p16 resulted in tumor growth reduction and increased survival (99).

In recent years, recombinant adenoviral vectors have showed better results in terms of efficiency of infection, easier and cheaper production and reduced risk (101). A large number of groups have addressed studies on *p16^{INK4a}*-based therapeutic strategies using replicative-deficient adenoviral vectors on a wide panel of cell lines and *in vivo* models (84, 86, 102-125) (Table I). All the *in vitro* studies agreed that adenovirally transferred p16INK4a induced cell cycle arrest and inhibition of proliferation in p16-deficient tumor cell lines. Several groups showed that cell cycle arrest induced by p16^{INK4a} was dependent on hypophosphorylation of pRb and on endogenous p16^{INK4a} and pRb status. However, some studies reported p16INK4a-induced suppression of growth even in p16-positive tumor cells (103, 125) or suggested pRb-independent activity (102, 122). In other studies, *pRb* was downregulated by *p16^{INK4a}* reexpression (81, 106, 107). Interestingly, many reports showed induction of apoptosis by exogenous p16^{INK4a} in a number of models (103, 104, 107, 111-113, 118, 120, 125). In some cases, p16-induced apoptosis was p53-dependent and accompanied cell cycle arrest (111, 112). In contrast, other groups reported induction of apoptosis in p53-deficient tumor cells (81, 103, 117). A remarkable set of results was obtained using glioma cell lines. In this model, reexpression of *p16^{INK4a}* resulted in suppression of invasion, inhibition of colony formation in soft agar and downregulation of VEGF. These *in vitro* studies are often accompanied by *in vivo* models to test the ability of adenovirally transferred *p16^{INK4a}* to stop tumor growth. Almost all the studies *in vivo* showed therapeutic activity that ranged from suppression of tumorigenicity and delay in tumor growth to tumor regression (86, 102-104, 107, 108, 116, 117, 119, 120, 122). In summary, these results support the use of adenovirus-*p16^{INK4a}* as a feasible and efficient therapeutic tool for cancer gene therapy.

One of the lessons learned in the development of *p53*-based cancer gene therapy strategies is that combined modality approaches can lead to much higher antitumoral activity than single agent treatments due to cooperative or synergistic effects. Restoration of p53-dependent

Table I: Summary of results obtained in gene therapy approaches involving reintroduction of the p16INK4a gene by means of retroviral (Retro-p16) or adenoviral (Ad-p16) recombinant vectors.

Agent	Model	*In vitro*	*In vivo*	Ref.
Retro-*p16*	Glioma	Cell cycle arrest and inhibition of proliferation	Tumor growth reduction and increased survival	99
	Breast cancer	Reduced growth, accumulation in G_1	Reduced tumorigenicity	100
	Esophageal SCC	pRb hypophosphorylation and senescence-like state		90
	T-ALL & AML	Cell cycle arrest and inhibition of proliferation	Suppression of lethal dissemination in syngeneic mice	96
		pRb-dependent inhibition of proliferation		98
		Inhibition of proliferation in $p16^{-/-}$ cell lines		97
Ad-*p16*	Different cell lines	Apoptosis; pRb-dependent cell cycle arrest	Reduced tumorigenicity and delay in tumor growth	120
		pRb-dependent cell cycle arrest		105
		Transcriptional downregulation of pRb		106
		Cell cycle arrest and inhibition of proliferation		110
	NSCLC	p53-casp3-dependent apoptosis		112
		Apoptosis associated with Bcl2 downregulation		111
		Cell cycle arrest and inhibition of growth; reduced colony formation		115
	Lung adenocarcinoma	High doses induce apoptosis		118
	Glioma	Suppression of invasion		84
		Inhibition of proliferation	Suppression of tumor growth	116
		Cell cycle arrest; suppression of colony formation		109
		Downregulation of VEGF expression	Inhibition of angiogenesis	86
	Pancreatic cancer	Inhibition of cell proliferation		114
		Cell cycle arrest and senescence; p53-independent apoptosis	Inhibition of tumor growth	103
	Colon cancer	p53-independent apoptosis	Prolonged survival and reduced growth	123
	Mesothelioma	Cell cycle arrest and cell death; decrease in pRb levels	Suppression of tumorigenicity and tumor regression	107
			Prolonged survival and potential cure	108
	Breast cancer	Cell cycle arrest; apoptosis	Inhibition of growth	104
		Apoptosis in $p16^{-/-}$ cell lines		113
	Prostate cancer	pRb-independent inhibition of proliferation	Inhibition of tumor growth; necrosis and fibrosis	102,122
	Ovarian cancer	pRb-dependent cell cycle arrest	Prolonged survival and reduced growth	124, 117
		p16-status independent arrest, reduced growth and apoptosis		125
	Head and neck cancer	Cell cycle arrest and inhibition of proliferation	Reduction or stabilization of tumor growth	119
	Esophageal SCC	Cell cycle arrest and inhibition of proliferation		121

apoptosis pathways, for instance, may enhance the effect of DNA-damaging chemotherapeutic drugs (93, 126).

Combination strategies involving adenovirus-mediated *p16INK4a* reintroduction have also been tested (Table II). For example, combined transfer of Ad-*p16* and Ad-*p53* into a panel of tumor cell lines resulted in cooperation to induce apoptosis (81). Similar approaches on ovarian cancer and pancreatic tumor cell lines (117, 127), and on xenografted human pancreatic tumors established in nude mice (127) showed additive but not cooperative effects. Given that $p16^{INK4a}$ induces p53-dependent and p53-independent apoptosis, future work should attempt to clarify this possible cooperation and to identify new cross talk mechanisms between $p16^{INK4a}$ and p53 pathways. Another interesting approach to amplify the cell cycle inhibitory activity of p16 is the coexpression of Cip/Kip genes. In an *in vitro* model of head and neck cancer, adenoviral cotransfer of *p16INK4a* and *p21Cip1* resulted in cell cycle arrest and inhibition of growth, but neither additive nor synergistic effects were observed(128). More successful was the construction of a *p16INK4a-p27Kip1* chimera, a fusion protein containing active functional parts of each CKI (129, 130). Adenovirus-mediated transference of *p16-p27* chimera (Ad-W9) showed p53- and pRb-independent apoptotic activity on tumor cell lines from different origins. Treatment of preestablished tumors resulted in tumor growth suppression and some cases of tumor regression, showing more potent antitumoral activity than the parental p16 and p27 molecules (131).

Table II: Summary of results obtained in gene therapy approaches involving adenovirus-mediated reintroduction of $p16^{INK4a}$ (Ad-p16) in combination with other therapeutic agents.

Agent	Model	*In vitro*	*In vivo*	Ref.
Ad-*p16*/Ad-*p53*	Different cell lines	Cell cycle arrest and decrease of pRb; cooperation in the induction of apoptosis with p53		81
	Pancreatic cancer	Cell cycle arrest and apoptosis	Tumor growth suppression	127
	Ovarian cancer	Growth inhibition and apoptosis alone or in combination with p53		117
Ad-*p16*/Ad-*p21*	Head and neck cancer	Cell cycle arrest and inhibition of growth Neither additive nor synergistic effects		128
Ad-p16-p27	Different cell lines	p53-independent tumor cell apoptosis	Tumor regression or tumor growth suppression	131
	Neointimal hyperplasia		Inhibition of injury-induced intimal hyperplasia	130
Ad-*p16*/ Ad-*GM-CFS*	Renal carcinoma		Inhibition of tumor growth	94
Ad-*p16*/Ad-*as-uPAR*	Glioma	$\alpha_v\beta_3$ downregulation		132
and Ad-*p16/as-uPAR*		Suppression of growth and invasion, $\alpha_v\beta_3$ downregulation	Suppression of growth (*ex vivo* and xenografts)	133
Ad-*p16*/ Chemotherapy	Glioma	Cell cycle arrest and chemoresistance to CDDP and ACNU		137
		Cell cycle arrest and chemoresistance to paclitaxel and topotecan		139
	Pancreatic cancer	Cell cycle arrest-deppendent chemoresistance to gemcitabine		141
	Ovarian cancer	Chemoresistance to taxanes and vinca alkaloids		138
	Bladder cancer	pRb-dependent cell cycle arrest and chemoresistence to CDDP and paclitaxel		136
	Osteosarcoma	Cell cycle arrest and chemoresistence to etoposide, or enhancement of cytotoxicity, depending on schedule		140
Ad-*p16*/	NSCLC	p53-dependent radiosensitization		135
radiotherapy	Lung adenocarcinoma	Increased radiosensitivity		134

Another new and promising approach to the treatment of glioma, a highly invasive tumor, was attempted by combining the reexpression of $p16^{INK4a}$ and the silencing of urokinase-type plasminogen activator receptor (uPAR) by means of a bicistronic construct ($p16^{INK4a}$ cDNA and uPAR antisense cassette). Infection of glioma cell lines with Ad-p16-asuPAR resulted in downregulation of $\alpha_v\beta_3$ and suppression of growth and invasion (132). Moreover, this combination suppressed tumor growth in *ex vivo* and *in vivo* glioma models (133).

As mentioned above, combination therapies using Ad-p53 and radiotherapy or chemotherapeutic drugs have showed important antitumoral activities. Following the same model, approaches involving Ad-*p16* and radio- or chemotherapy have been tested. Reexpression of $p16^{INK4a}$ can induce radiosensitization *in vitro* (134, 135). On the other hand, all the reports in the literature agree that *p16*-induced cell cycle arrest results in decreased sensitivity to classical chemotherapeutic agents, such as CDDP (136, 137), taxanes (136, 138, 139), etoposide (140), gemcitabine (141), nimustine (ACNU) (137), vinca alkaloids (138) and topotecan (139). To note, one group reported increased sensitivity to etoposide when $p16^{INK4a}$ was reexpressed after drug treatment (140), indicating that p16 could participate in a DNA damage checkpoint in a manner similar to p53. In any case, the use of $p16^{INK4a}$ function to protect healthy tissues from the pejorative effects of chemotherapy, especially when treating p16-irresponsive tumors, should be considered (142).

Specific CDK inhibitor molecules

Finally, another option to therapeutically recover proliferation control is the use of specific CDK inhibitors that may mimic CKI function. In recent years several active-site inhibitors of CDK have been tested (roscovitine, olomoucine, staurosporine, UCN-01, *etc.*), but these ATP antagonists generally presented low specificity for CDK

versus other kinases. Some of these inhibitors have showed antitumor activity *in vitro*, but were associated with a high toxicity even for normal nontransformed cells. However, screening of collections of compounds, as well as rational design based on enzyme-ligand complex crystal structures, are now yielding preclinical candidates, particularly certain purine and flavonoid analogues, with impressive potency and selectivity (143). For instance, some CDK4/6 specific inhibitors have been found in screening for molecules able to induce pRb-dependent cell cycle arrest (3-ATA), followed by *in vitro* kinase assays to assess their specificity (144). The resolution of crystal structures of CDK-ligand complexes allows the design of synthetic molecules with high affinity for the active site or for the macromolecular substrate binding site (145-147). Similarly, it is also possible to target other required domains in cyclins or CDKs, such as regulation sites, cyclin-binding sites, cellular localization domain and destruction box. Finally, the design of small peptides that mimic binding and inhibitory functions of CKI proteins is one of the most promising approaches to the therapeutic targeting of CDK activity and cell proliferation. Lane and coworkers designed a small peptide of 20 amino acids able to reproduce the CDK inhibitory properties of p16. When linked to a carrier peptide (from the drosophila antennapedia homeodomain), this oligopeptide entered the cells and induced pRB hypophosphorylation and cell cycle arrest (148, 149). Moreover, this peptide inhibited $\alpha_v\beta_3$ integrin mediated cell spreading and migration (83), showing the participation of G_1 CDK in integrin regulatory pathways that control matrix-dependent cell growth and migration. The antitumoral activity of a similar antennapedia-p16 peptide has been demonstrated in a model of pancreatic cancer both *in vitro* (150) and *in vivo*.

Together with therapeutic potential, these new drugs and peptides are expected to have a relevant role in future research attempting to understand the biological functions of INK4 and CDK4/6 proteins. In addition, the feasibility of these molecules to be systemically administered can overcome the limitations in the delivery of gene therapy agents.

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