

# p14<sup>ARF</sup>: Role in the cellular stress response and applications to cancer

## Review Article

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**Abbreviations:** mouse embryo fibroblasts, (MEFs); Hypoxia-inducible factor-1, (HIF-1); retinoblastoma tumor suppressor, (pRb); exon 1 -encoded region of human ARF, (ARF1); cis-diamminedichloroplatinum, (CDDP);

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## Summary

The p14<sup>ARF</sup> tumor suppressor (ARF) plays a central role in human cancer, as evidenced by its loss of function in up to 40% of cancers overall, and by the ability of ectopic ARF expression to suppress growth and induce cell death in tumor cells *in vitro* and *in vivo*. Deletion of ARF or deletion of the N-terminal region of ARF encoded by its first exon (exon 1) in mice results in a predisposition to tumor formation. An important and well-described activity of ARF is enhancing p53 stability by binding to the mdm2 protein, and blocking mdm2-mediated ubiquitination and degradation of p53. Loss of ARF results in unopposed mdm2-mediated degradation of p53, thereby attenuating the endogenous p53 pathway of growth arrest and apoptosis. Loss of ARF may attenuate the activity of ectopically expressed p53 as well, compromising the outcome of p53 gene therapy and other p53-based therapies for cancer. ARF also appears to have tumor suppressor function independent of the p53/mdm2/ARF regulatory mechanism, and has been observed to interact with a variety of proteins other than mdm2. Taken together, the combined activities of ARF are likely to play important combined roles in the underlying mechanism of cancer and provide new opportunities to refine and optimize gene based strategies for a wide variety of cancers.

## I. Introduction

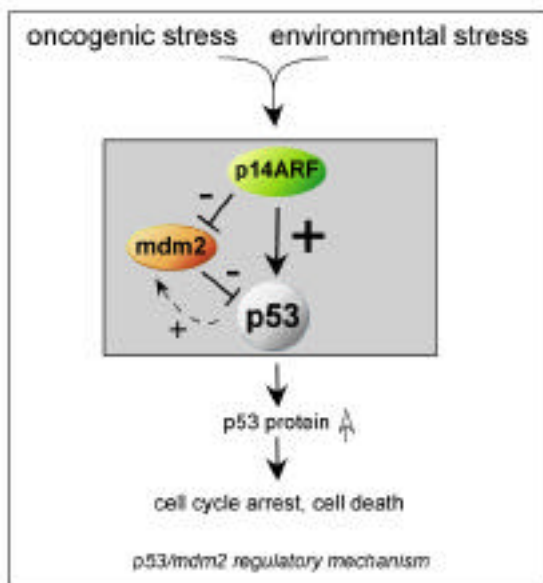
ARF plays a key role in the p53/mdm2 stress response mechanism that is central to cancer. This mechanism serves as a point of convergence for a variety of stress signals encountered by the cancer cell, including environmental stress (radiation, exposure to chemotherapeutic drugs, hypoxia), and endogenous oncogenic stress (oncogene activation, genome instability and the ensuing DNA damage) (Shieh et al, 1997; Siliciano et al, 1997; Bates et al, 1998; de Stanchina et al, 1998; Palmero et al, 1998; Zindy et al, 1998; Gjerset et al, 1999). In response to these signals, the mechanism promotes the accumulation of p53 protein, followed by induction of p53 downstream events leading to cell cycle arrest or apoptosis. We now know that this mechanism is disrupted in virtually all cancers (see below), usually through deletion or mutation of p53 in more than 50% of cancers (Levine, 1997), or through deletion or promoter methylation of ARF in about 40% of cancers (Gruis et al, 1995; Fulci et al, 2000; Pinyol et al, 2000). Disruption of this mechanism impairs the cancer cell's ability to trigger cell cycle arrest or apoptosis in response to oncogenic abnormalities, enabling it to manifest its transformed

phenotype, or in response to environmental stress, which can contribute to drug resistance. The high frequency with which this mechanism is targeted for disruption in cancer suggests that an intact mechanism cannot be easily bypassed and is incompatible with cancer cell growth. The mechanism provides therefore an important point of focus for therapeutics development, as treatments that restore a fully functioning p53/mdm2 regulatory mechanism would be expected to be highly suppressive of most cancers, while having minimal consequences for normal cells that lack the endogenous oncogenic stress signals that feed into the mechanism. In fact, ectopic overexpression of wild-type p53 is highly suppressive of cell growth and viability of most cancer cells (Cai et al, 1993) but is minimally suppressive of normal epithelial cells and fibroblasts (Katayose et al, 1995). Even in cancer cells that retain expression of endogenous wild-type p53 and would likely have defects in other components of the regulatory mechanism, overexpressed ectopic wild-type p53 can have suppressive effects on growth and viability (Saadatmandi et al, 2002a). P53 gene therapy has therefore been investigated as a promising targeted approach to cancer treatment, applicable to a variety of cancers. Several clinical applications of p53 gene therapy have now entered

Phase III trials (Saadatmandi et al, 2002b). With the identification of ARF as a key player in modulating p53 activity, and possibly a critical determinant of p53 activity, it is likely that further refinements in this approach will be possible.

**Figure 1** diagrams our current understanding of the interactions between ARF, mdm2 and p53. ARF regulates the interaction of p53 with mdm2, a key negative regulator of p53 (Pomerantz et al, 1998; Zhang et al, 1998). Mdm2 (also termed hdm2 in humans) ubiquitinates p53 and targets it for proteasome-mediated degradation. Through this mechanism, mdm2 ensures low steady state levels of wild-type p53 found in normal cells. Mdm2 expression in turn is induced by p53, establishing a feedback loop that controls expression levels of both proteins (Wu et al, 1993). Mdm2 function appears to be largely p53-dependent as mdm2 overexpression in transgenic mice results in embryonic lethality, but lethality can be rescued by p53 deletion (Jones et al, 1995; Montes de Oca Luna et al, 1995). Mdm2 overexpression occurs in about 10% of cancers, predominantly in soft tissue sarcomas (Leach et al, 1993; Cordon-Cardo et al, 1994). Such tumors generally continue to express wild-type p53, as would be expected if overexpression of mdm2 were the functional equivalent of p53 loss.

ARF interacts physically with mdm2, through the first 20 residues of the N-terminal domain of ARF and the C-terminal domain of mdm2 (Midgley et al, 2000). The interaction opposes mdm2-mediated degradation of p53, and leads to an increase in p53 half-life (Kamijo et al, 1998), enhanced sequence-specific transcriptional transactivation by p53 (Kamijo et al, 1997; Pomerantz et al, 1998), and p53-mediated cell cycle arrest (Zhang et al, 1998). Nucleolar sequestration of mdm2 by ARF has been proposed as important in this process (Tao and Levine, 1999). ARF expression is induced by oncogenic abnormalities such as oncogene activation (de Stanchina et al, 1998; Palmero et al, 1998; Zindy et al, 1998) and is



**Figure 1.** Schematic representation of the stress-activated regulatory loop involving p53, mdm2, and p14ARF.

necessary to induce p53-mediated cell cycle arrest in response to these abnormalities. The observation that p53 and ARF abnormalities often occur in a reciprocal manner in human cancer is consistent with this p53-dependent model for ARF activity and suggests that ARF loss is to some extent the functional equivalent of p53 loss (Pomerantz et al, 1998; Zhang et al, 1998). However, unlike mdm2, ARF function does not appear to be confined to a largely p53-dependent role, and is now known to interact with proteins other than mdm2, some of which may mediate important tumor suppressor activities of ARF.

## II. p53-independent activities of ARF

In addition to its well-described involvement in regulating p53 activity, several lines of evidence now support the involvement of p53-independent activities of ARF in tumor suppression. In some cancers, for example, ARF loss and p53 mutation occur together (Gazzeri et al, 1998; Sanchez-Cespedes et al, 1999), suggesting that inactivation of ARF per se constitutes an independent survival advantage for the cancer cell. Evidence for a p53-independent role for ARF *in vivo* is also suggested by studies of knockout mice, where ARF-mediated suppression of spontaneous tumor formation in p53-null, mdm2-null mice was inferred based on the decreased predisposition of such mice to spontaneous tumor formation compared to mice lacking ARF as well as p53 and mdm2 (Weber et al, 2000). This effect is also observed *in vitro*, where mouse embryo fibroblasts (MEFs) from mice triply nullizygous for p53, mdm2, and ARF are growth suppressed by ectopic re-expression of ARF, indicating that ARF is able to suppress growth independently of p53 in this setting (Weber et al, 2000). It has been speculated that in such a setting, ARF may interact with mdmx (discussed below) or another mdm2 homologue to reverse their inhibition of a yet to be identified growth suppressor (Jackson et al, 2001).

Another study using MEFs implicated both the Rb and p53 pathways in ARF activity (Carnero et al, 2000). In that study MEFs lacking the p53 pathway could be suppressed by ectopic expression of ARF, but MEFs lacking both p53 and Rb pathways could not be suppressed by ectopic expression of ARF (Carnero et al, 2000). In our own studies in human tumor cell lines, we see that ectopic overexpression of ARF suppresses growth and viability of human tumor cells lacking expression of endogenous wild-type p53 (Saadatmandi et al, 2002a; Huang et al, 2003). Unlike tumor cells expressing endogenous wild-type p53, ectopic overexpression of ARF in tumor cells lacking expression of endogenous wild-type p53 does not lead to p53 accumulation, or to the induction of p53 target gene expression (mdm2, p21waf1, bax), or to an elevated bax to bcl2 ratio (Saadatmandi et al, 2002a). ARF is therefore able to suppress tumor cell growth independently of these key players in p53-dependent growth arrest and apoptosis. Furthermore, since the tumor lines studied lacked a functional Rb pathway as well (Saadatmandi et al, 2002a), ARF appears to have tumor suppressor activity in human tumor cells that is independent of both p53 and Rb.

ARF binding proteins other than mdm2 have been identified through yeast two-hybrid screening techniques, co-immunoprecipitation, and co-localization studies. These proteins, listed in **Table 1**, include topoisomerase I (Karayan et al, 2001), E2F-1,2,3 (Eymin et al, 2001; Martelli et al, 2001), spinophilin, the regulatory subunit of protein phosphatase I (Vivo et al, 2001), p120<sup>E4F</sup>, a zinc finger transcription repressor (Rizos et al, 2003), CARF, a novel collaborator of ARF (Hasan et al, 2002), mdmx, a mdm2 homologue (Jackson et al, 2001), and HIF-1, a hypoxia-inducible transcription factor (Fatyol and Szalay, 2001). ARF has also been found to form oligomers with itself (Menendez et al, 2003). Two of the ARF binding proteins listed in **Table 1**, mdmx and HIF-1, may participate in p53 independent functions of ARF. Hypoxia-inducible factor-1 (HIF-1), a transcriptional regulator of genes induced during hypoxia, is frequently over expressed in advanced tumors and may play an important role in tumor growth. ARF can inhibit the

transcriptional activity of HIF-1 by binding to it and sequestering it into the nucleolus. The interaction between ARF and HIF-1 is independent of both mdm2 and p53, and thus may define a p53 independent role for ARF (Fatyol and Szalay, 2001). Mdmx has been implicated in the p53-dependent activity of ARF, participating through interactions with mdm2, ARF and p53, in the p53/mdm2 regulatory feedback loop, and inhibiting transcriptional transactivation by p53 (Jackson and Berberich, 2000). Through its association with ARF, mdmx appears to interfere with ARF-mdm2 interactions (Jackson et al, 2001). In the presence of wild-type p53, and under conditions of oncogenic stress where p53 and mdm2 levels are induced, mdmx may play a minor role.

However, under conditions where p53 and mdm2 are low or absent (i.e., triple knockout MEFS discussed above, or possibly certain human tumor lines), associations between mdmx and ARF may mediate tumor suppressor activities independently of p53 (Jackson et al, 2001).

**Table 1.** p14ARF binding proteins

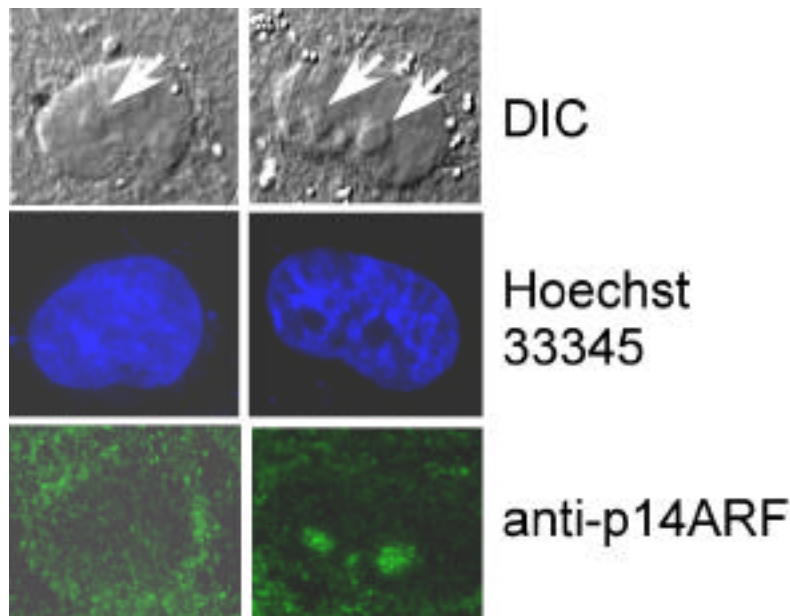
Protein name	How identified	Protein type	activity	reference
<b>P120<sup>E4F</sup></b>	Yeast 2-hybrid	Zinc finger transcription repressor	Forms ternary complex with p53 and ARF; enhances cell cycle inhibition	(Rizos et al., 2003)
<b>E2F-1, E2F-2, E2F-3</b>	IP-Western	Transcription factors that induce S-phase	ARF binds to and destabilized them	(Eymin et al., 2001; Martelli et al., 2001)
<b>spinophilin</b>	Yeast 2-hybrid	Regulatory subunit of protein phosphatase I catalytic protein	Synergizes with ARF to suppress cell growth	(Vivo et al., 2001)
<b>Topoisomerase I</b>	IP-Western	DNA binding	DNA repair, DNA synthesis; ARF enhances its activity	(Karayan et al., 2001)
<b>mdmx</b>	Co-localization; IP-Western	Mdm2 homologue	Blocks p53 Binds mdm2 Binds ARF and opposes it	(Jackson et al., 2001)
<b>HIF-1</b>	IP-Western	Hypoxia-inducible transcription factor	ARF blocks HIF-1 ; sequester to nucleolus; p53-independent activity	(Fatyol and Szalay, 2001)
<b>Pex19p</b>	Yeast 2-hybrid	Farnesylated cytosolic protein	Interacts with mouse ARF, NOT human ARF	(Sugihara et al., 2001; Wadhwa et al., 2002)
<b>CARF</b>	Yeast 2-hybrid	Novel serine-rich protein "Collaborator of ARF"	Enhances ARF activity	(Hasan et al., 2002)
<b>P14ARF</b>	Western	Tumor suppressor	Forms homo-oligomers	(Menendez et al., 2003)

### III. Functional significance of the ARF C-terminus

The ARF tumor suppressor is encoded by the *ARF-INK4a* locus of chromosome 9p21, a region that has already been implicated in human cancer as a very frequent target of genetic alteration (Kamb et al, 1994). Also encoded by this locus is the p16 (MTS1/CDKN2) tumor suppressor, an inhibitor of cyclin D-dependent kinases CDK4 and CDK6 (Serrano et al, 1993). Phosphorylation of the retinoblastoma tumor suppressor (pRb) by CDK4 or CDK6 releases the E2F transcription factor from an inactive complex with pRb, and enables downstream events that promote cell cycle progression. Inactivation of either p16 or pRb deregulates cell cycle progression, a key feature of tumor cell metabolism. While p16 and ARF are structurally and functionally unrelated and have distinct first exons (denoted exon 1 and exon 1', respectively), they are characterized by the unusual property of sharing the same exon 2, although alternate reading frames are used to generate the two proteins.

Studies on murine ARF (p19) have localized the p53-dependent tumor suppressor activity of ARF to the N-terminal exon 1' encoded sequences, which are completely non-overlapping with p16 sequences (Zhang et al, 1998). In murine ARF the regions important for nucleolar localization, mdm2 sequestration, and p53-dependent apoptosis reside entirely within the first 37 N-terminal residues and these 37 residues are sufficient to carry out these effects (Weber et al, 2000). With regard to this activity, the C-terminal sequences might appear therefore to constitute a largely non-functional and dispensable domain. Because the N-terminal exon 1'-encoded sequences of murine and human ARF are highly conserved (particularly in the first 14 amino acids

involved in mdm2 binding), it seemed likely that this conclusion would extend to human ARF as well. However, while the N-terminal exon 1'-encoded region of human ARF (ARF1) appears sufficient to bind mdm2 and to mediate the p53-dependent activity of ARF, several studies now raise the possibility that ARF C-terminal sequences, encoded by ARF exon 2, also contribute to its tumor suppressor properties. For example, human ARF has nucleolar localization sequences in both N terminal (exon 1') and C-terminal (exon 2) domains (Weber et al, 1999; Rizos et al, 2000), and both may be required for efficient nucleolar localization (Zhang and Xiong, 1999). An example of the differences in subcellular distribution of ARF and ARF1 is shown in **Figure 2** (Y Huang and R Gjerset, unpublished). MCF-7 breast cancer cells, which express endogenous wild-type p53 and lack endogenous expression of ARF were treated with replication defective adenoviral vectors encoding ARF1 or full length ARF (vectors described in (Saadatmandi et al, 2002a; Huang et al, 2003). 48 hours post-vector treatment cells were fixed and stained with anti-p14ARF (Zymed laboratories) and goat anti-rabbit Alexa 488 (Molecular Probes) plus Hoechst 33345. The Hoechst stain binds DNA and facilitates the identification of nuclei and subnuclear structures. The results show that over expressed nuclear ARF is concentrated in nucleoli (**Figure 2**, first column, bottom panel), consistent with published studies (Rizos et al, 2001). In contrast, nuclear ARF1 showed far less nucleolar concentration and distributed more evenly throughout the nucleus (**Figure 2**, second column, bottom panel). In addition, several studies on melanoma-prone kindreds suggest the involvement of ARF C-terminal sequences in ARF functions (Rizos et al, 2001; Hashemi et al, 2002). In contrast to an earlier study that reported that



**Figure 2.** Subcellular distribution of ectopic ARF and ARF1 $\beta$  in MCF7 breast cancer cells (endogenous ARF null). Cells were treated with adenoviral vectors encoding ARF1 $\beta$  or full length p14ARF, followed 24 hours later by fixing and staining. Nucleoli, indicated by arrows in top panels, are evident by DIC (Differential Interference Contrast) and by Hoechst staining as darker regions (middle panels). Bottom panels show immunofluorescent staining for p14ARF. Magnification = 60x.

several cancer associated mutations affecting C-terminal (exon 2-encoded) sequences altered p16 function but not ARF function (Quelle et al, 1997), the melanoma study identified mutations in the C-terminal nucleolar localization domain encoded by exon 2 in certain melanoma-prone kindreds that altered the subcellular distribution of ARF and/or its binding to mdm2 (Rizos et al, 2001). Another study also reported a C-terminal mutation in ARF in a melanoma kindred that gave rise to a protein carrying an 8 residue deletion that was less potent than wild-type ARF at stabilizing p53 and inducing cell cycle arrest (Hashemi et al, 2002). Interestingly, the physical properties of these mutants were often altered as well, as several of them (R81Q, R82L, R88ter) showed greater solubility in the absence of urea than did full length wild-type ARF (Rizos et al, 2001). C-terminal sequences therefore contribute to the proper folding of ARF and conceivably influence the intermolecular interactions and activity of ARF.

ARF C-terminal sequences have now been shown to be crucial for ARF interaction with topoisomerase I, for the ability of ARF to stimulate topoisomerase I relaxation activity, and for the co-localization of ARF and topoisomerase I in the nucleolus (Olivier et al, 2003). Furthermore, two ARF C-terminal point mutations that are highly conserved among species were unable to stimulate topoisomerase I activity (Olivier et al, 2003). Taken together the data argue for an important role for the ARF C-terminal sequences in regulating the structure and function of ARF, although the reason why p14ARF and p16 overlap remains unknown.

#### IV. Perspectives for clinical translation

The disruption of the p53/p14ARF/mdm2 feedback loop is likely to be an essential survival mechanism common to most cancers, as it renders cancer cells unable to undergo growth arrest or apoptosis in response to the intracellular oncogenic stress signals that are universal features of cancer. One would predict that restoration of normal functioning of the loop would be highly suppressive of cancer cell growth and viability, in that it exploits the cancer cell's intrinsic fragility and predisposition to apoptosis, but would have minimal consequence for normal cells that lack the endogenous oncogenic stress signals that feed into the loop. The importance of ARF in regulating this loop, and the high frequency with which ARF function is lost in cancer, makes ARF an important therapeutic target.

Numerous therapeutic applications of the p53 tumor suppressor are now in an advanced stage of development, with clinical trials ongoing or completed that attest to the safety and efficacy of adenovirus-mediated gene transfer of p53 for a variety of tumors, including prostate cancer, lung cancer, and head and neck cancer, brain cancer, ovarian cancer, and bladder cancer (see references (Logothetis, 1999; Swisher et al, 1999; Nemunaitis et al, 2000; Sweeney and Pisters, 2000; Swisher and Roth, 2000)). The vector of choice for these applications has been a replication defective vector based on adenovirus

serotype 5, a serotype that lacks serious pathogenicity, has a broad host cell range, displays a high infection efficiency for most cell types, and is relatively easy to prepare in high yield and high titer, and is stable. Unlike retroviral vectors, adenoviral genomes do not integrate into the host cell DNA, thereby avoiding the risk of insertional mutagenesis, which is a concern with retroviral vectors. To minimize potential vector toxicity and to maximize the exposure of the target tumor cells to the vector, these trials have generally employed direct administration of the vector into a patient's tumor or into the region where a tumor is localized, with doses as high as  $10^{11}$  plaque forming units per injection being well tolerated (Saadatmandi et al, 2002b). While patients may develop neutralizing antibody to the vector, expression of the p53 gene in tumors is still detectable, and antibody does not preclude multiple administrations of vector at weekly or biweekly intervals (Saadatmandi et al, 2002b). An extension of such approaches to include ARF therefore seems feasible, and approaches that exploit both p53 and ARF may prove to be far more effective than approaches based on p53 alone.

The ultimate challenge for cancer treatment is metastatic disease, for which systemic delivery approaches are required. Systemic delivery of p53 using viral (Lebedeva et al, 2001) or non viral (Ramesh et al, 2001) delivery strategies can have antitumor efficacy in mice and reduce the incidence of metastatic spread when combined with conventional therapy. However, the clinical application of systemic delivery approaches will likely require the development of improved second generation viral or non viral vectors, including vectors consisting of polyethylenimine (PEI)-DNA complexes (Densmore et al, 2001), and possibly incorporating a tumor specific targeting moiety to improve delivery efficiency, a critical factor in the success of any gene transfer strategy. Because systemic gene delivery is likely to be far less efficient than direct intratumoral gene delivery, there is also a need to optimize gene combinations so as to improve therapeutic efficacy at low gene expression levels.

#### V. Combined potential of p53 and ARF

The activity of p53 is attenuated through a feedback mechanism whereby p53 induces the expression of its own inhibitor, mdm2 (**Figure 1**). This feedback mechanism, which may be exacerbated by certain cancer associated abnormalities such as loss of p14ARF or overexpression of mdm2, may compromise the outcome of p53-based strategies for cancer, including p53 gene transfer. Full activation of the pathway may therefore require additional steps to modulate the activity of mdm2. One approach is to supply both p53 and p14ARF, either as a combination of single gene vectors (Tango et al, 2002) or as a bicistronic vector (Huang et al, 2003). Co-expression of p14ARF and p53 leads to a dramatic enhancement of tumor suppression compared to that achieved with p53 alone, as a result of increased accumulation of p53 protein followed by increased expression of p53 target genes, including mdm2, p21waf1, and bax, an elevated bax to bcl2 ratio, and

apoptosis (Huang et al, 2003). P14ARF also appears to promote increased recruitment of p53 message into polysomes and increased synthesis of p53 protein, through a mechanism that is not yet understood (Huang et al, 2003) and Huang and Gjerset, unpublished). The cooperative effects of ectopically expressed p53 and p14ARF are observed even in cells expressing endogenous ARF, indicating that the presence of endogenous ARF is likely to be insufficient to counterbalance the effects of induced levels of mdm2. The use of an optimized gene combination involving p53 together with p14ARF, could therefore enhance efficacy under conditions where gene delivery was inefficient or resulted in low levels of transgene expression.

Eventually, small molecule approaches targeting specific molecular structures or interactions may provide alternatives to gene delivery when systemic delivery is needed for metastatic disease. One possibility is the use of peptides corresponding to regions of the p53 protein or to segments of p53-binding proteins so as to correct the folding of p53 mutant proteins and reactivate function (Selivanova et al, 1998; Lane and Lain, 2002). A better understanding of how ARF contributes to the pathology of cancer, including a mapping of regions necessary for function and for interaction with itself and other proteins may make it possible to design ARF peptide mimetics or other small molecule therapeutics, to mimic protein-protein interactions critical for ARF activity, or disrupt interactions that impede its activity (Menendez et al, 2003). Such molecules could be administered systemically, possibly tagged with moieties to facilitate cellular uptake (Ho et al, 2001).

## VI. ARF contributes to the p53-mediated DNA damage response

A variety of anticancer agents, including cisplatin (cis-diamminedichloroplatinum, CDDP) and doxorubicin (adriamycin), generate lesions that promote p53-mediated apoptosis, and that these agents are more effective *in vitro* and *in vivo* in animal tumor models, if p53 function is also restored in these tumors (Clarke et al, 1993; Lotem and Sachs, 1993; Lowe et al, 1993; Gjerset et al, 1995; Nguyen et al, 1996; Dorigo et al, 1998; Gjerset and Mercola, 2000; Gjerset et al, 2001; Lebedeva et al, 2001; Saadatmandi et al, 2002b). There is also evidence that loss or mutation of p53 in cancer contributes to therapy resistance (O'Connor et al, 1997), currently the major obstacle to successful treatment of cancer. The fact that many chemotherapeutic agents use the p53 pathway to kill their target cells has now been exploited in clinical trials of several cancers, including advanced head and neck cancer, where p53 gene transfer was seen to improve responses to cisplatin or radiation (Swisher et al, 1999; Nemunaitis et al, 2000; Swisher and Roth, 2000; Saadatmandi et al, 2002b). These observations are consistent with the well-described involvement of p53 in the DNA damage response (Bakalkin et al, 1995; Lee et al, 1995), which leads to phosphorylation of mdm2 and disruption of the interaction between p53 and mdm2. In this way, p53 is stabilized and activated as a transcription factor, followed by induction

of downstream target genes involved in growth arrest (p21waf1) or apoptosis (bax) (Levine, 1997). P53-mediated senescence, or irreversible loss of proliferative potential, has also been associated with the outcome of therapy, and correlates with increased expression of senescence-associated  $\beta$ -galactosidase (Campisi, 2001; Schmitt et al, 2002).

In light of the important role played by ARF in the opposing mdm2-mediated degradation of p53, it is likely that ARF gene transfer will be required to optimize the outcome of p53-chemotherapy combination approaches. In fact, deletion of the INK4A/ARF locus in E $\mu$ -myc murine lymphoma cells impairs apoptosis, compromises p53 function, and reduces the response to the DNA alkylating agent cyclophosphamide *in vitro* and *in vivo* (Schmitt et al, 1999). Other studies have shown that ARF expression, though dispensable for initiating a p53 response to DNA damage, is required for a sustained accumulation of p53 following DNA damage (Khan et al, 2000). ARF gene transfer alone can sensitize breast cancer cells to doxorubicin, a drug that acts in part through the induction of DNA damage (Guo-Chang and Chu-Tse, 2000), or to cisplatin, which forms bifunctional adducts on DNA (Deng et al, 2002). A role for ARF in the cellular response to these chemotherapeutic agents would greatly expand its potential application to cancer treatment, as it could be used to help reverse therapy resistance in advanced cancers.

## VII. Conclusions

ARF has emerged as a critical human tumor suppressor with relevance to the underlying mechanism of cancer and to cancer treatment. Through its involvement in the p53/mdm2 feedback loop, which shuts down cell growth in response to oncogenic abnormalities, ARF plays a key role in suppressing a broad array of cancers that retain wild-type p53 expression. ARF may also be necessary to optimize p53-based therapies and to fully activate the p53-mediated response to conventional chemotherapy. In addition, through pathways that are independent of p53 and less well understood, but which presumably involve ARF interactions with proteins other than mdm2, ARF may contribute additional tumor suppressor activities that add to its p53-dependent activity. As we gain a better understanding of these activities and how they are regulated we will certainly improve and refine our paradigms to understand cancer and find new opportunities for the development of highly targeted cancer therapeutics.

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## References

- Bakalkin G, Selivanov G, Yakovleva T, Kiseleva E, Kashuba E, Magnusson Kp, Szekely L, Klein G, Terenius L, Wiman K (1995) p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. **Nucleic Acids Res** 23, 362-369.
- Bates S, Phillips Ac, Clark P, Stott F, Peters G, Ludwig R, Vousden K (1998) p14ARF links the tumour suppressors RB and p53. **Nature** 395, 124-125.
- Cai D, Mukhopadhyay T, Liu Y, Fujiwara T, Roth J (1993) Stable expression of the wild-type p53 gene in human lung cancer cells after retrovirus-mediated gene transfer. **Hum Gene Ther** 4, 617-624.
- Campisi J (2001) Cellular senescence as a tumor-suppressor mechanism. **Trends Cell Biol** 11, S27-31.
- Carnero A, Hudson J, Price Cm, Beach D (2000) p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. **Nat Cell Biol** 2, 148-155.
- Clarke A, Purdie C, Harrison D, Morris R, Bird C, Hooper M, Wyllie A (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways [see comments]. **Nature** 362, 849-852.
- Cordon-Cardo C, Latres E, Drobnjak M, Oliva M, Pollack D, Woodruff J, Marechal V, Chen J, Brennan M, Levine A (1994) Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. **Cancer Res** 54, 794-799.
- De Stanchina E, Mccurrach M, Zindy F, Shieh S, Ferbeyre G, Samuelson A, Prives C, Roussel M, Sherr C, Lowe S (1998) E1A signaling to p53 involves the p19(ARF) tumor suppressor. **Genes Dev** 12, 2434-2442.
- Deng X, Kim M, Vandier D, Jung Y, Rikiyama T, Sgagias M, Goldsmith M, Cowan K (2002) Recombinant adenovirus-mediated p14(ARF) overexpression sensitizes human breast cancer cells to cisplatin. **Biochem Biophys Res Commun** 296, 792-798.
- Densmore C, Kleiner E, Gautam A, Jia S, Xu B, Worth L, Waldrep J, Fung Y, Tang A, Knight V (2001) Growth suppression of established human osteosarcoma lung metastases in mice by aerosol gene therapy with PEI-p53 complexes. **Cancer Gene Ther** 8, 619-627.
- Dorigo O, Turla S, Lebedeva S, Gjerset R (1998) Sensitization of rat glioblastoma multiforme to cisplatin in vivo following restoration of wild-type p53 function. **J Neurosurg** 88, 535-540.
- Eymin B, Karayan L, Seite P, Brambilla C, Brambilla E, Larsen Cj, Gazzeri S (2001) Human ARF binds E2F1 and inhibits its transcriptional activity. **Oncogene** 20, 1033-1041.
- Fatyal K, Szalay A (2001) The p14ARF tumor suppressor protein facilitates nucleolar sequestration of hypoxia-inducible factor-1alpha (HIF-1) and inhibits HIF-1-mediated transcription. **J Biol Chem** 276, 28421-28429.
- Fulci G, Labuhn M, Maier D, Lachat Y, Hausmann O, Hegi M, Janzer R, Merlo A, Van Meir E (2000) p53 gene mutation and ink4a-arf deletion appear to be two mutually exclusive events in human glioblastoma. **Oncogene** 19, 3816-3822.
- Gazzeri S, Della Valle V, Chaussade L, Brambilla C, Larsen C, Brambilla E (1998) The human p19ARF protein encoded by the beta transcript of the p16INK4a gene is frequently lost in small cell lung cancer. **Cancer Res** 58, 3926-3931.
- Gjerset R, Haghghi A, Lebedeva S, Mercola D (2001) Gene therapy approaches to sensitization of human prostate carcinoma to cisplatin by adenoviral expression of p53 and by antisense jun kinase oligonucleotide methods. **Methods Mol Biol** 175, 495-520.
- Gjerset R, Lebedeva S, Haghghi A, Turla S, Mercola D (1999) Inhibition of the Jun kinase pathway blocks DNA repair, enhances p53-mediated apoptosis and promotes gene amplification. **Cell Growth Differ** 10, 545-554.
- Gjerset R, Mercola D (2000) Sensitization of tumors to chemotherapy through gene therapy. **Adv Exp Med Biol** 465, 273-291.
- Gjerset R, Turla S, Sobol R, Scalise J, Mercola D, Collins H, Hopkins P (1995) Use of wild-type p53 to achieve complete treatment sensitization of tumor cells expressing endogenous mutant p53. **Mol Carcinog** 14, 275-285.
- Gruis N, Weaver-Feldhaus J, Liu Q, Frye C, Eeles R, Orlow I, Lacombe L, Ponce-Castaneda V, Lianes P, Latres E, et al. (1995) Genetic evidence in melanoma and bladder cancers that p16 and p53 function in separate pathways of tumor suppression. **Am J Pathol** 146, 1199-1206.
- Guo-Chang F, Chu-Tse W (2000) Transfer of p14ARF gene in drug-resistant human breast cancer MCF-7/Adr cells inhibits proliferation and reduces doxorubicin resistance. **Cancer Lett** 158, 203-210.
- Hasan M, Yaguchi T, Sugihara T, Kumar P, Taira K, Reddel R, Kaul S, Wadhwa R (2002) CARF is a novel protein that cooperates with mouse p19ARF (human p14ARF) in activating p53. **J Biol Chem** 277, 37765-37770.
- Hashemi J, Lindstrom M, Asker C, Platz A, Hansson J, Wiman K (2002) A melanoma-predisposing germline CDKN2A mutation with functional significance for both p16 and p14ARF. **Cancer Lett** 180, 211-221.
- Ho A, Schwarze S, Mermelstein S, Waksman G, Dowdy S (2001) Synthetic protein transduction domains: enhanced transduction potential in vitro and in vivo. **Cancer Res** 61, 474-477.
- Huang Y, Tyler T, Saadatmandi N, Lee C, Borgstrom P, Gjerset R (2003) Enhanced tumor suppression by a p14ARF/p53 bicistronic adenovirus through increased p53 protein translation and stability. **Cancer Res** 63, 3646-3653.
- Jackson M, Berberich S (2000) MdmX protects p53 from Mdm2-mediated degradation. **Mol Cell Biol** 20, 1001-1007.
- Jackson M, Lindstrom M, Berberich S (2001) MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. **J Biol Chem** 276, 25336-25341.
- Jones S, Roe A, Donehower L, Bradley A (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. **Nature** 378, 206-208.
- Kamb A, Gruis N, Weaver-Feldhaus J, Liu Q, Harshman K, Tavitgian S, Stockert E, Day R, 3rd, Johnson B, Skolnick M (1994) A cell cycle regulator potentially involved in genesis of many tumor types [see comments]. **Science** 264, 436-440.
- Kamijo T, Bodner S, Van De Kamp E, Randle D, Sherr C (1999) Tumor spectrum in ARF-deficient mice. **Cancer Res** 59, 2217-2222.
- Kamijo T, Weber J, Zambetti G, Zindy F, Roussel M, Sherr C (1998) Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. **Proc Natl Acad Sci U S A** 95, 8292-8297.
- Kamijo T, Zindy F, Roussel M, Quelle D, Downing J, Ashmun R, Grosveld G, Sherr C (1997) Tumor suppression at the mouse Ink4a locus mediated by the alternative reading frame product p19ARF. **Cell** 91, 649-659.
- Karayan L, Riou J, Seite P, Migeon J, Cantereau A, Larsen C (2001) Human ARF protein interacts with topoisomerase I and stimulates its activity. **Oncogene** 20, 836-848.
- Katayose D, Gudas J, Nguyen H, Srivastava S, Cowan K, Seth P (1995) Cytotoxic effects of adenovirus-mediated wild-type p53 protein expression in normal and tumor mammary epithelial cells. **Clin Cancer Res** 1, 889-897.
- Khan S, Moritsugu J, Wahl G (2000) Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, ribonucleotide depletion. **Proc Natl Acad Sci U S A** 97, 3266-3271.
- Lane D, Lain S (2002) Therapeutic exploitation of the p53 pathway. **Trends Mol Med** 8, S38-42.

- Leach F, Tokino T, Meltzer P, Burrell M, Oliner J, Smith S, Hill D, Sidransky D, Kinzler K, Vogelstein B (1993) p53 Mutation and MDM2 amplification in human soft tissue sarcomas. **Cancer Res** 53, 2231-2234.
- Lebedeva S, Bagdasarova S, Tyler T, Mu X, Wilson D, Gjerset R (2001) Tumor Suppression and Therapy Sensitization of Localized and Metastatic Breast Cancer by Adenovirus p53. **Hum Gene Ther** 12, 763-772.
- Lee S, Elenbaas B, Levine A, Griffith J (1995) p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. **Cell** 81, 1013-1020.
- Levine A (1997) p53, the cellular gatekeeper for growth and division. **Cell** 88, 323-331.
- Logothetis C, et al. (1999) AdCMV-p53 intraprostatic gene therapy preceding radical prostatectomy (RP): an in vivo model for targeted therapy development. **J. Clin. Oncol.** 18, 1203A.
- Lotem J, Sachs L (1993) Hematopoietic cells from mice deficient in wild-type p53 are more resistant to induction of apoptosis by some agents. **Blood** 82, 1092-1096.
- Lowe S, Ruley H, Jacks T, Housman D (1993) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. **Cell** 74, 957-967.
- Martelli F, Hamilton T, Silver D, Sharpless N, Bardeesy N, Rokas M, Depinho R, Livingston D, Grossman S (2001) p19ARF targets certain E2F species for degradation. **Proc Natl Acad Sci U S A** 98, 4455-4460.
- Menendez S, Khan Z, Coomber D, Lane D, Higgins M, Koufali M, Lain S (2003) Oligomerization of the human ARF tumor suppressor and its response to oxidative stress. **J Biol Chem** 278, 18720-18729.
- Midgley C, Desterro J, Saville M, Howard S, Sparks A, Hay R, Lane D (2000) An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. **Oncogene** 19, 2312-2323.
- Montes de oca Luna R, Wagner D, Lozano G (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. **Nature** 378, 203-206.
- Nemunaitis J, Swisher S, Timmons T, Connors D, Mack M, Doerksen L, Weill D, Wait J, Lawrence D, Kemp B, Fossella F, Glisson B, Hong W, Khuri F, Kurie J, Lee J, Lee J, Nguyen D, Nesbitt J, Perez-Soler R, Pisters K, Putnam J, Richli W, Shin D, Walsh G, et al. (2000) Adenovirus-mediated p53 gene transfer in sequence with cisplatin to tumors of patients with non-small-cell lung cancer. **J Clin Oncol** 18, 609-622.
- Nguyen D, Spitz F, Yen N, Cristiano R, Roth J (1996) Gene therapy for lung cancer: enhancement of tumor suppression by a combination of sequential systemic cisplatin and adenovirus-mediated p53 gene transfer. **J Thorac Cardiovasc Surg** 112, 1372-1376; discussion 1376-1377.
- O'Connor P, Jackman J, Bae I, Myers T, Fan S, Mutoh M, Scudiero D, Monks A, Sausville E, Weinstein J, Friend S, Fornace Aj, Jr., Kohn K (1997) Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. **Cancer Res** 57, 4285-4300.
- Olivier A, Lucie K, Jean-Francois R, Christian-Jacques L, Paule S (2003) Delineation of the domains required for physical and functional interaction of p14ARF with human topoisomerase I. **Oncogene** 22, 1945-1954.
- Palmero I, Pantoja C, Serrano M (1998) p19ARF links the tumour suppressor p53 to Ras. **Nature** 395, 125-126.
- Pinyol M, Hernandez L, Martinez A, Cobo F, Hernandez S, Bea S, Lopez-Guillermo A, Nayach I, Palacin A, Nadal A, Fernandez P, Montserrat E, Cardesa A, Campo E (2000) INK4a/ARF locus alterations in human non-Hodgkin's lymphomas mainly occur in tumors with wild-type p53 gene. **Am J Pathol** 156, 1987-1996.
- Pomerantz J, Schreiber-Agus N, Liegeois N, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee H, Cordon-Cardo C, Depinho R (1998) The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. **Cell** 92, 713-723.
- Quelle D, Cheng M, Ashmun R, Sherr C (1997) Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. **Proc Natl Acad Sci U S A** 94, 669-673.
- Ramesh R, Saeki T, Smyth Templeton N, Ji L, Stephens L, Ito I, Wilson D, Wu Z, Branch C, Minna J, Roth J (2001) Successful Treatment of Primary and Disseminated Human Lung Cancers by Systemic Delivery of Tumor Suppressor Genes Using an Improved Liposome Vector. **Mol Ther** 3, 337-350.
- Rizos H, Darmanian A, Holland E, Mann G, Kefford R (2001) Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF. **J Biol Chem** 276, 41424-41434.
- Rizos H, Darmanian A, Mann G, Kefford R (2000) Two arginine rich domains in the p14ARF tumour suppressor mediate nucleolar localization. **Oncogene** 19, 2978-2985.
- Rizos H, Diefenbach E, Badhwar P, Woodruff S, Becker T, Rooney R, Kefford R (2003) Association of p14ARF with the p120E4F transcriptional repressor enhances cell cycle inhibition. **J Biol Chem** 278, 4981-4989.
- Saadatmandi N, Tyler T, Huang Y, Haghghi A, Frost G, Borgstrom P, Gjerset R (2002a) Growth suppression by a p14(ARF) exon 1beta adenovirus in human tumor cell lines of varying p53 and Rb status. **Cancer Gene Ther** 9, 830-839.
- Saadatmandi N, Wilson D, Gjerset R (2002b) p53 Gene Therapy. In Encyclopedia of Cancer. (Academic Press) pp. 425-432.
- Sanchez-Cespedes M, Reed A, Buta M, Wu L, Westra W, Herman J, Yang S, Jen J, Sidransky D (1999) Inactivation of the INK4A/ARF locus frequently coexists with TP53 mutations in non-small cell lung cancer. **Oncogene** 18, 5843-5849.
- Schmitt C, Fridman J, Yang M, Lee S, Baranov E, Hoffman R, Lowe S (2002) A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. **Cell** 109, 335-346.
- Schmitt C, Mccurrach M, De Stanchina E, Wallace-Brodeur R, Lowe S (1999) INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. **Genes Dev** 13, 2670-2677.
- Selivanova G, Kawasaki T, Ryabchenko L, Wiman K (1998) Reactivation of mutant p53: a new strategy for cancer therapy. **Semin Cancer Biol** 8, 369-378.
- Serrano M, Hannon G, Beach D (1993) A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4 [see comments]. **Nature** 366, 704-707.
- Shieh S, Ikeda M, Taya Y, Prives C (1997) DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. **Cell** 91, 325-334.
- Siliciano J, Canman C, Taya Y, Sakaguchi K, Appella E, Kastan M (1997) DNA damage induces phosphorylation of the amino terminus of p53. **Genes Dev** 11, 3471-3481.
- Sugihara T, Kaul S, Kato J, Reddel R, Nomura H, Wadhwa R (2001) Pex19p dampens the p19ARF-p53-p21WAF1 tumor suppressor pathway. **J Biol Chem** 276, 18649-18652.
- Sweeney P, Pisters L (2000) Ad5CMVp53 gene therapy for locally advanced prostate cancer--where do we stand? **World J Urol** 18, 121-124.

- Swisher S, Roth J (2000) Gene Therapy in Lung Cancer. **Curr Oncol Rep** 2, 64-70.
- Swisher S, Roth J, Nemunaitis J, Lawrence D, Kemp B, Carrasco C, Connors D, El-Naggar A, Fossella F, Glisson B, Hong W, Khuri F, Kurie J, Lee J, Lee J, Mack M, Merritt J, Nguyen D, Nesbitt J, Perez-Soler R, Pisters K, Putnam J, Jr., Richli W, Savin M, Waugh M, et al. (1999) Adenovirus-mediated p53 gene transfer in advanced non-small-cell lung cancer. **J Natl Cancer Inst** 91, 763-771.
- Tango Y, Fujiwara T, Itoshima T, Takata Y, Katsuda K, Uno F, Ohtani S, Tani T, Roth J, Tanaka N (2002) Adenovirus-mediated p14ARF gene transfer cooperates with Ad5CMV-p53 to induce apoptosis in human cancer cells. **Hum Gene Ther** 13, 1373-1382.
- Tao W, Levine A (1999) P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. **Proc Natl Acad Sci U S A** 96, 6937-6941.
- Vivo M, Calogero R, Sansone F, Calabro V, Parisi T, Borrelli L, Saviozzi S, La Mantia G (2001) The human tumor suppressor arf interacts with spinophilin/neurabin II, a type 1 protein-phosphatase-binding protein. **J Biol Chem** 276, 14161-14169.
- Wadhwa R, Sugihara T, Hasan M, Taira K, Reddel R, Kaul S (2002) A major functional difference between the mouse and human ARF tumor suppressor proteins. **J Biol Chem** 277, 36665-36670.
- Weber J, Jeffers J, Rehg J, Randle D, Lozano G, Roussel M, Sherr C, Zambetti G (2000) p53-independent functions of the p19(ARF) tumor suppressor. **Genes Dev** 14, 2358-2365.
- Weber J, Taylor L, Roussel M, Sherr C, Bar-Sagi D (1999) Nucleolar Arf sequesters Mdm2 and activates p53. **Nat Cell Biol** 1, 20-26.
- Wu X, Bayle J, Olson D, Levine A (1993) The p53-mdm-2 autoregulatory feedback loop. **Genes Dev** 7, 1126-1132.
- Zhang Y, Xiong Y (1999) Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. **Mol Cell** 3, 579-591.
- Zhang Y, Xiong Y, Yarbrough W (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. **Cell** 92, 725-734.
- Zindy F, Eischen C, Randle D, Kamijo T, Cleveland J, Sherr C, Roussel M (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. **Genes Dev** 12, 2424-2433.



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