[Cell Cycle 4:3, 465-472; March 2005]; ©2005 Landes Bioscience

Report

Enforced Expression of p14ARF Induces p53-Dependent Cell Cycle Arrest but Not Apoptosis

Stuart Gallagher
Richard F. Kefford
Helen Rizos*

Westmead Institute for Cancer Research; University of Sydney at Westmead Millennium Institute; Westmead Hospital; Westmead, Australia

*Correspondence to: Helen Rizos; Westmead Institute for Cancer Research; University of Sydney at Westmead Millennium Institute; Westmead Hospital; Westmead NSW 2145, Australia; Tel.: +61.2.9845.9509; Fax: +61.2.9845.9102; Email: helen_rizos@wmi.usyd.edu.au

Received 12/19/04; Accepted 01/07/05

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/abstract.php?id=1526

KEY WORDS

p14ARF, p53, apoptosis, melanoma

ABBREVIATIONS

IPTG	isopropyl β-D-thiogalactopyranoside
MEF	mouse embryonic fibroblast
DRB	5,6-Dichlorobenzimidazole ribosode
PI	propidium iodide
LM	light microscopy

ACKNOWLEDGEMENTS

We thank Dr. Eileen McGowan for generating the U20S_ARF cell clone and Dr. Tom Shenk for supplying the pCMVEGFP-spectrin plasmid.

H.R. is a National Health and Medical Research Council RD Wright Fellow, University of Sydney. This work was supported by the National Health and Medical Research Council, the University of Sydney, and the NSW Health Department.

ABSTRACT

Expression of the p14ARF tumour suppressor is induced by hyperproliferative signals produced by RAS, MYC and other oncogenes. p14ARF quenches inappropriate mitogenic signaling by activating the p53 pathway, and the frequent loss of p14ARF in human cancer diminishes the duration and level of the p53 response. Consistent with this role, p14ARF accumulation can induce potent cell cycle arrest, but its role in promoting apoptosis has not been well established. Therefore we investigated the effects of p14ARF on the survival and growth of several human cell types. To avoid the toxicity associated with adenoviral-based vectors, we established inducible expression of p14ARF in p53-intact and p53-deficient human cell lines. As expected, transient and inducible expression of p14ARF induced rapid cell cycle arrest only in tumour cells expressing intact p53. Further, p14ARF expression did not promote apoptosis in primary human fibroblasts, or in any human tumour cell line tested, irrespective of p53 status. Instead, p14ARF expression sensitized cells to apoptosis in the presence of inhibitors of topoisomerase II (adriamycin) and transcription (DRB). Thus, loss of p14ARF would be an important step in the selection of apoptotic resistant tumour cells.

INTRODUCTION

The *INK4a/ARF* locus on chromosome 9 is inactivated in a large proportion of human tumours and inherited mutations involving this locus are associated with melanoma predisposition in 20–40% of multiple case melanoma families.[1,2] This unusual locus encodes two products, $p16^{INK4a}$ and p14ARF, from alternatively spliced transcripts, in different reading frames. Constitutional and somatic *INK4a/ARF* mutations specifically targeting $p16^{INK4a}$ are common in cancer,[3-6] and less frequent alterations exclusively inactivating p14ARF have also been found in human tumours and familial melanoma kindreds.[7-9] Moreover, approximately 50% of the somatic mutations identified in human cancer affect the amino acid sequence of both $p16^{INK4a}$ and p14ARF,[6] and high-density mapping on chromosome 9p in human melanomas has identified *p14ARF* as the most commonly deleted *INK4a/ARF* gene.[10] The mechanisms of ARF action are therefore critical to understanding the genesis and tumour progression of melanoma, and the precise modifying effects of ARF on cell growth and survival may have major importance to the chemo- and radiation resistance that confounds effective treatment of advanced melanoma in humans.

p14ARF interacts with the p53 ubiquitin ligase, hdm2 to prevent the nuclear export and degradation of p53.[11-14] ARF is induced by hyperproliferative signals produced by Ras, c-MYC, E2F-1 and other oncogenes.[15-18] Consequently, the loss of ARF limits the response of the p53 network to hyperproliferative signals, and reduces the duration of p53 activity in response to DNA damaging stimuli.[19,20]

Although some reports suggest that p14ARF function requires p53,[21] there is evidence indicating that ARF also possesses p53-independent functions. The murine ARF homologue, p19ARF, can suppress colony formation of p53-null cells by affecting the pRb pathway,[22] and mice lacking p53 and p19ARF develop a wider spectrum of tumours than animals lacking either tumour suppressor alone.[23] Furthermore, inactivation of p19ARF and p53 was not mutually exclusive in immortalized mouse embryonic fibroblasts (MEFs).[22] Similarly, various human tumours exhibit simultaneous loss of p53 and ARF,[24,25] and p14ARF has been reported to limit S-phase progression and colony formation in p53-null human cancer cells.[26-28] p19ARF can also inhibit the processing of ribosomal RNA,[29] and the murine and/or human ARF protein interact with other regulators including the E2F transcription factors,[30,31] spinophilin, topoisomerase I, cyclin G, MdmX,

HIF-1α, pex19p, CARF, p120^{E4F}, B23, tat-binding protein and Werner's helicase.[32-41] Apart from hdm2 and E2F-1, however, none of the other ARF-interacting proteins have been shown to directly mediate the effects of ARF on cell cycle progression.[22,42]

ARF expression quenches inappropriate mitogenic signalling by forcing cells to undergo growth arrest or apoptosis, depending on the cellular context. Enforced ARF expression in MEFs induces cell cycle arrest,[43] whereas cells overexpressing p19ARF, together with E1A, Myc or RAS oncogenes undergo apoptosis.[16,44,45] Activation of ARF alone can also trigger apoptosis; the ectopic expression of ARF from adenoviral vectors induced apoptosis in p53-null MEFs, p53-intact human U20S cells, and p53-deficient human Saos-2 cells.[46] The C-terminal region of the murine ARF protein (amino acids 130–169) is important for p19ARF-mediated apoptosis, but this region is not conserved in human p14ARF.[47] Thus, p14ARF may not be as potent an apoptotic regulator as its murine counterpart. This may explain the lack of extensive apoptosis in human diploid fibroblasts overexpressing Myc, despite obvious upregulation of p14ARF and p53.[48] Some reports have shown that adenoviral expression of p14ARF did not promote apoptosis in p53-null human cells,[21,26,46,49] but induced cell death in p53-intact cells.[26,47,50-52] In contrast, in another study p14ARF induced apoptosis independent of p53.[53]

In order to clarify the apoptotic function of the human p14ARF tumour suppressor we established inducible expression of p14ARF in p53-intact and p53-deficient human cell lines. Using these cell models the impact of long-term p14ARF induction on cell proliferation and cell death was thoroughly examined. In addition we investigated the role of transient p14ARF expression in normal human fibroblasts and human tumour cells. Our data show that expression of p14ARF alone, promoted rapid and potent cell cycle arrest in a p53-dependent manner, but did not induce cell apoptosis. We also show that p14ARF expression sensitized cells to the pro-apoptotic effects of adriamycin and DRB (5,6-dichlorobenzimidazole ribosode).

MATERIALS AND METHODS

Cell culture. Human U2OS osteosarcoma cells (ARF-null, p53 wild type), Saos-2 osteosarcoma cells (ARF wild type, p53-null), HCT116 colorectal cancer cells (ARF-mutant, p53

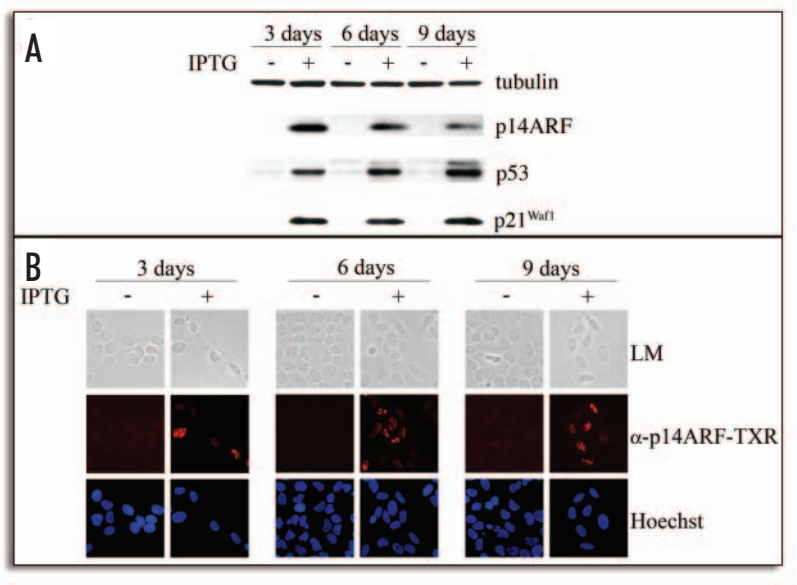

Figure 1. Induced p14ARF expression activates p53. (A) Expression of p14^{ARF}, p53, p21^{Waf1} and tubulin was determined three, six or nine days after treatment of U20S_ARF cells with 1mM IPTG (+) or PBS (-). (B) The nucleolar accumulation of p14ARF in IPTG treated U20S_ARF cells was detected by immunofluorescence. Hoechst staining was used to visualize cell nuclei. LM, light microscopy.

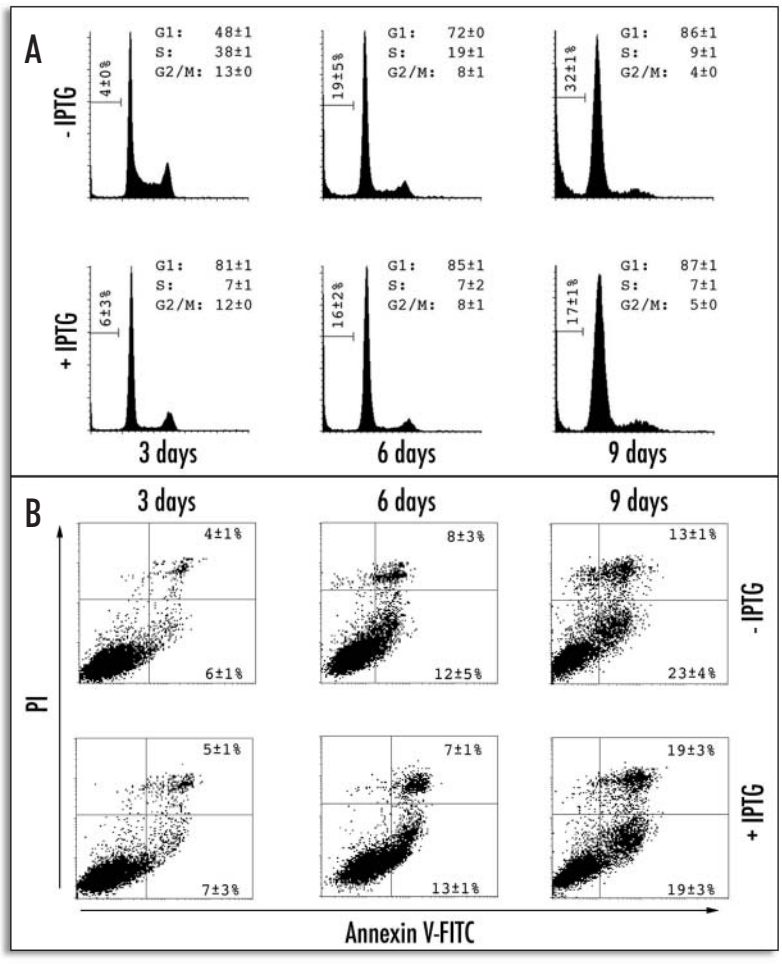

Figure 2. p14ARF induces rapid G_1 cell cycle arrest but no apoptosis. (A) The cell cycle distribution of U20S_ARF cells was examined three, six and nine days post IPTG induction, using propidium iodide staining. Each result is obtained from at least two independent experiments. (B) Dual colour flow cytometric Annexin V analysis for apoptosis of U20S_ARF cells treated with 1 mM IPTG (+) or PBS (-), for three, six or nine days. Cells that are Annexin V-positive and PI-negative are in early apoptosis and cells positive for both Annexin V and PI dye are in the late stages of apoptosis. Each results is obtained from at least two independent experiments. PI, propidium iodide.

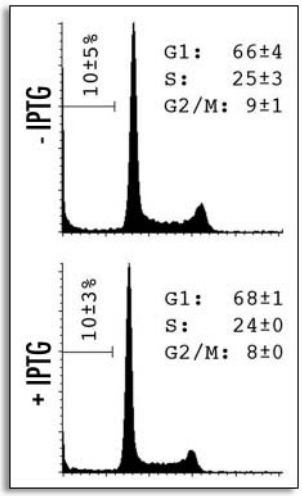

Figure 3. IPTG does not alter cell cycle distribution. The influence of 1 mM IPTG on the cell cycle distribution of the U20S lac repressor parental cell line was determined by flow cytometric analysis of propidium iodide stained nuclei, 72 h post treatment.

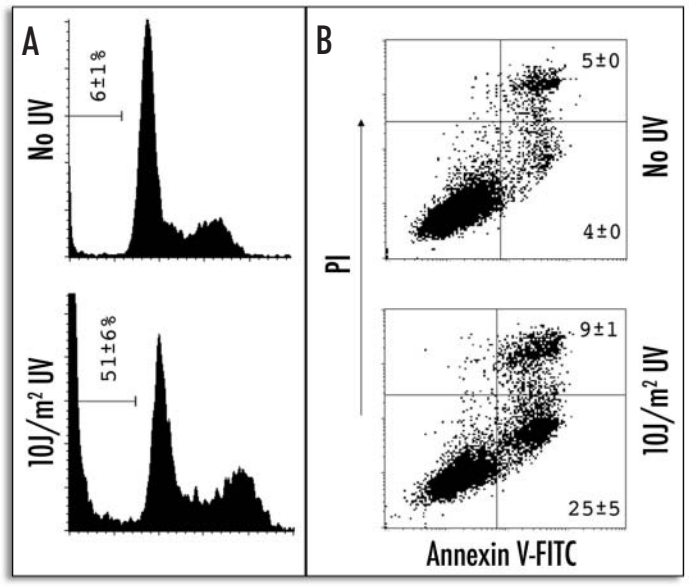

Figure 4. Analysis of apoptosis in the U20S_ARF cells. The apoptotic response of the U20S_ARF cell line was examined three days after exposure to 10J/m^2 UVC irradiation. The cell cycle distribution of propidium iodide stained nuclei was examined by flow cytometry (A), and apoptosis was measured using annexin V staining (B). PI, propidium iodide.

wild-type), WMM1175 melanoma cells (ARF-null, p53-null[54]) and WS1 primary human skin fibroblasts (ATCC) were grown in Dulbecco's modified Eagle's medium (Trace Scientific, Sydney) supplemented with 10% fetal bovine serum and glutamine. All cells were cultured in a 37°C incubator with 5% CO_2.

The IPTG (isopropyl β-D-thiogalactopyranoside)-inducible mammalian expression system (Lac-switch system; Stratagene) was used to obtain U2OS_ARF and WMM1175_ARF cell clones carrying the stably integrated *p14ARF* gene under IPTG-inducible expression control. Full-length *p14ARF* cDNA was cloned into the *POPRSVICAT* expression plasmid, and transfected using Lipofectamine 2000 (Invitrogen), into a WMM1175 and U2OS clone expressing the lac repressor. Transfected cells were selected with hygromycin (200 μg/ml; Invitrogen) and geneticin (500 μg/ml; Invitrogen). Stable cell clones were seeded 24 hours prior to induction, and were induced with 1–5 mM IPTG, which was refreshed with a further 1–5 mM IPTG every three days.

In cytotoxicity assays, cells were exposed to media containing 1 μM adriamycin in the presence of absence of 1mM IPTG for 6 h. Adriamycin containing media was removed, cells rinsed with fresh media, and then incubated with media containing either PBS or 1mM IPTG for 72 h. Cells were exposed to 25 μM DRB in the presence or absence of IPTG for 72 h. U20S_ARF cells were also treated with the topisomerase I inhibitor, camptothecin (10 μM), in the presence of absence of 1 mM IPTG for 6 h. The media was then removed, and after washing the cells were incubated for a further 72 h in media containing either PBS or 1mM IPTG. Alternatively, cells were exposed to 1 μM camptothecin in the presence or absence of IPTG for 72 h. For UV-treatment, cells were irradiated at 254nm using a Stratalinker 2400 (Stratagene).

Transient cell transfections. For cell cycle distribution analysis of transiently transfected cells, cultured cells (1 x 10^6) were seeded in T75 flasks and cotransfected with the *p14ARF-FLAG5b* or *pCMV-FLAG5b* plasmid (6 μg) and *pCMVEGFP-spectrin* (2 μg) using Lipofectamine 2000 (Invitrogen).

Cell cycle and apoptosis analysis. Adherent and floating cells were combined and sub-divided into samples for cell cycle, cell death and protein expression analysis. For cell cycle analysis, cells were fixed in 70% ethanol at 4°C for at least 1 h, washed in PBS and stained with propidium iodide (50 ng/μl) containing ribonuclease A (50 ng/μl). In transient transfection assays, EGFP-spectrin was used as a marker for analysis of transfected cells. DNA content from at least 2000 cells was analyzed using ModFIT software. Numbers of cells with sub-G_1 content were determined using CellQuest software. Annexin-V staining was performed as detailed by the manufacture (Sigma).

Western blotting. Proteins were extracted for 1 h at 4°C using RIPA lysis buffer containing protease inhibitors (Roche). The protein concentration was determined with the Dc Protein Assay Kit (Bio-Rad). Proteins (30–50 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were probed with antibodies against p53 (D0-1, Santa Cruz), $p21^{Waf1}$ (C-19, Santa Cruz), p14ARF (DCS-240, Sigma) and tubulin (Molecular Probes).

Indirect immunofluorescence. Cultured cells (1 x 10^5) seeded on coverslips in six-well plates were washed in PBS and fixed in 3.7% formaldehyde. Cells were immunostained for 50 min with monoclonal mouse anti-p14ARF antibody (Sigma) followed by a 50 min exposure to Texas red-conjugated anti-mouse secondary IgG (Molecular Probes). Nuclei were visualized by Hoechst 33258 staining (2 μg/ml).

RESULTS

Inducible p14ARF promotes p53-dependent cell cycle arrest, but not apoptosis. To investigate the cell cycle regulatory role of p14ARF we established stable p14ARF-inducible clones in the p53-intact U20S cell line using the IPTG-inducible expression system. Expression of p14ARF was induced with 1mM IPTG over a nine day period. As shown in Figure 1A, strong expression of p14ARF was detected in U20S_ARF cell clones, three days post induction, and ARF expression was maintained up to nine days of continuous IPTG exposure. As expected, accumulation of p14ARF led to the stabilisation of p53 in the U20S_ARF cells over the nine day treatment, and induction of the p53-target $p21^{Waf1}$, confirmed that p14ARF induced the activation of p53 in these cells (Fig. 1A).

Immunostaining of the U20S_ARF cells after three, six and nine days of IPTG induction showed detectable nucleolar p14ARF in approximately 80% of cells (Fig. 1B). Using Hoechst 33258 staining we observed very few cells (less than 5%) showing the characteristic nuclear fragmentation associated with apoptotic cells, even after nine days of p14ARF induction.

Considering that apoptotic cells often detach from tissue culture flasks, we analysed the cell cycle distribution of adhered and floating cells using flow cytometry. As expected, induction of p14ARF in the p53-positive

U20S cells promoted potent G_1 cell cycle arrest and S-phase inhibition that was maintained throughout the nine day IPTG treatment (Fig. 2A). p14ARF-mediated arrest was less evident nine days after IPTG exposure, because the uninduced control U20S_ARF cells had reached confluency. Although p14ARF-induction promoted growth arrest in U20S_ARF cells, there was no increase in apoptosis, as measured by the sub-G_1 population (Fig. 2A). Only the control uninduced U20S cells showed a significant increase in sub-G_1 cell population at nine days post induction, again a result of reaching confluency (Fig. 2A). Similar data was obtained with Annexin-V staining; there was no significant difference in the percentage of apoptotic cells between induced and uninduced U20S_ARF cells over the nine day IPTG treatment period (Fig. 2B).

To ensure that IPTG was not affecting cell growth, we treated the parental U20S cell line (expresses the lac repressor but not p14ARF) with 1 mM IPTG for three days. As expected, IPTG treatment did not affect cell cycle distribution, or the level of cell death in this cell line (Fig. 3). We also confirmed the suitability of U20S_ARF cells for apoptotic studies by irradiating these cells with low levels (10 J/m^2) of UVC. Flow cytometric analysis of the U20S_ARF cells, 72 hours post irradiation, confirmed that UV induced significant apoptosis. Specifically, the sub-G_1 population increased from 6% to 51% (Fig. 4A) and the proportion of Annexin-V positive, apoptotic cells increased from 9% to 34% (Fig. 4B).

p14ARF requires intact p53 to inhibit cell cycle progression. Considering that p14ARF has been shown to induce apoptosis in a p53-independent manner, we investigated the impact of p14ARF expression on the growth of the p53-null melanoma cell line WMM1175. The p14ARF-inducible WMM1175_ARF cells were treated with 5 mM IPTG, and expression of p14ARF confirmed by Western blotting (Fig. 5A). As expected, in the absence of p53, p14ARF did not induce the expression of the CDK inhibitor, p21^{Waf1}. Nucleolar localisation of p14ARF in the WMM1175_ARF cells was also detected by immunostaining in approximately 50% of cells (data not shown). Although p14ARF was induced in this cell line, we observed no changes in the cell cycle distribution profile by flow cytometry (Fig. 5B), and there was no evidence of p14ARF-induced apoptosis, as determined by flow cytometry and Annexin-V staining, even nine days post treatment (Fig. 5C).

Transient p14ARF expression does not promote apoptosis. To exclude the possibility that genetic damage in our selected cell clones alters important apoptotic signalling pathways, we transiently introduced p14ARF into various human cells. We transfected Saos-2 osteosarcoma and HCT116 colorectal cancer cells, both of which have been shown to undergo apoptosis in the presence of recombinant adenovirus expressing p14ARF.[53] In addition we transiently introduced p14ARF into the WS1 human primary skin fibroblasts. These cells were transfected with p14ARF and harvested 48 hours later. Western blot analysis confirmed p14ARF expression in all transfected cells (Fig. 6A). In the p53-null Saos-2, no p53 was detected and levels of p21^{Waf1} did not change. Transient expression of p14ARF in Saos-2 cells caused a small increase in the G_1 phase (less than 10%), but there was no significant change in apoptosis (Fig. 6B). The transient expression of p14ARF in the p53-intact HCT116 cells caused an increase in the level of p53 and p21^{Waf1}, with the expected G_1-phase arrest, but no p14ARF-induced apoptosis (Fig. 6). Finally, ectopic expression of p14ARF had no impact on the cell cycle distribution of the slow growing primary human fibroblasts (94% of cells are already in G_1 phase), and p14ARF expression did not induce cell death (Fig. 6B).

p14ARF sensitizes human cells to apoptosis. Considering that p14ARF accumulation potently activated the p53 pathway in the U20S_ARF cells, we investigated whether p14ARF conferred increased sensitivity to apoptosis.

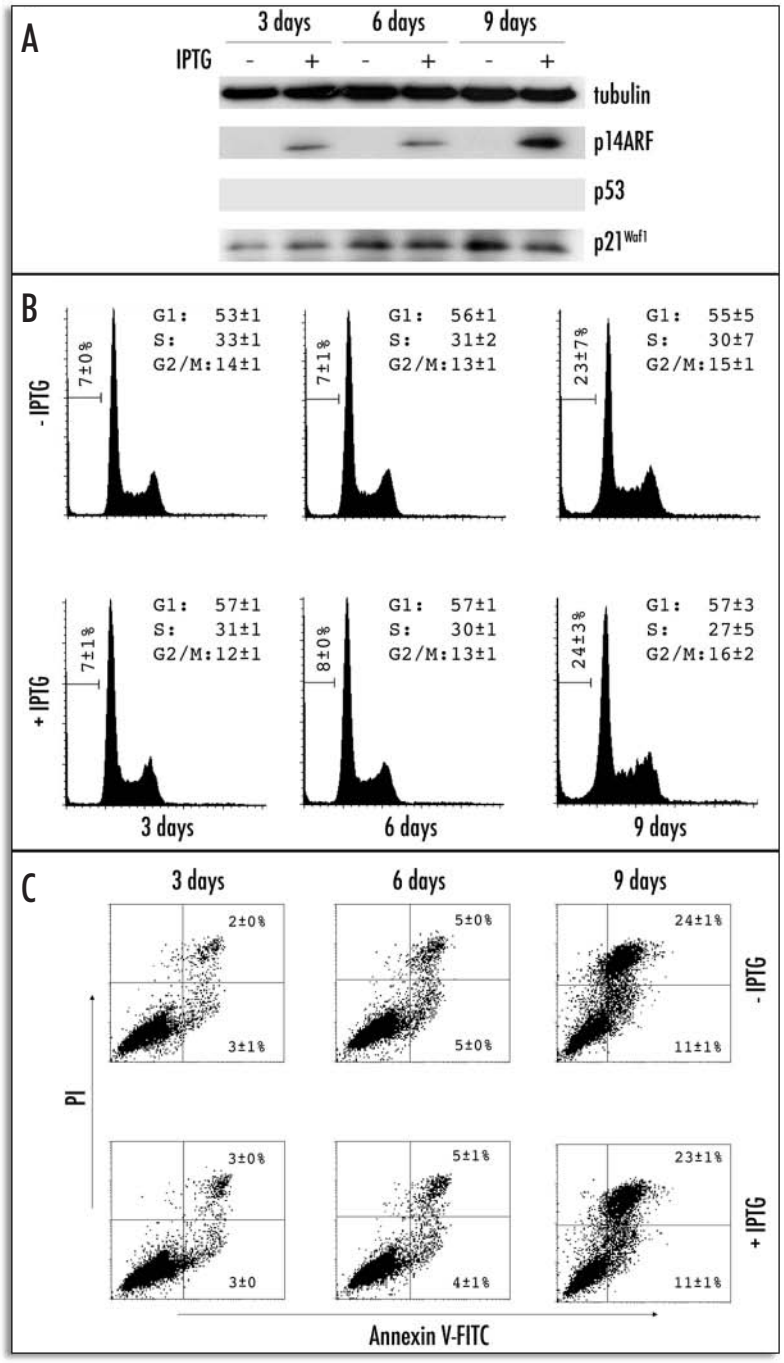

Figure 5. Induced expression of p14ARF does not promote cell cycle arrest or apoptosis in the p53-null WMM1175 melanoma cells. (A) Expression of p14ARF, p53, p21^{Waf1} and tubulin was determined three, six or nine days after treatment of WMM1175_ARF cells with 5mM IPTG (+) or PBS (-). (B) The cell cycle distribution of WMM1175_ARF cells was examined three, six and nine days post IPTG induction, using propidium iodide staining. Each result is obtained from at least two independent experiments. (C) Dual colour flow cytometric Annexin V analysis for apoptosis of WMM1175_ARF cells treated with 5mM IPTG (+) or PBS (-), for three, six or nine days. Each results is obtained from at least two independent experiments. PI, propidium iodide.

To test this hypothesis, induced and uninduced U20S_p14ARF cells were treated with clinically important inhibitors of topoisomerase I and topoisomerase II, camptothecin and adriamycin, respectively. In addition, U20S_p14ARF cells were also exposed to the casein kinase II and RNA synthesis inhibitor DRB. Short term (6 h) exposure of uninduced U20S_p14ARF cells to 1 μM adriamycin increased the endogenous levels of

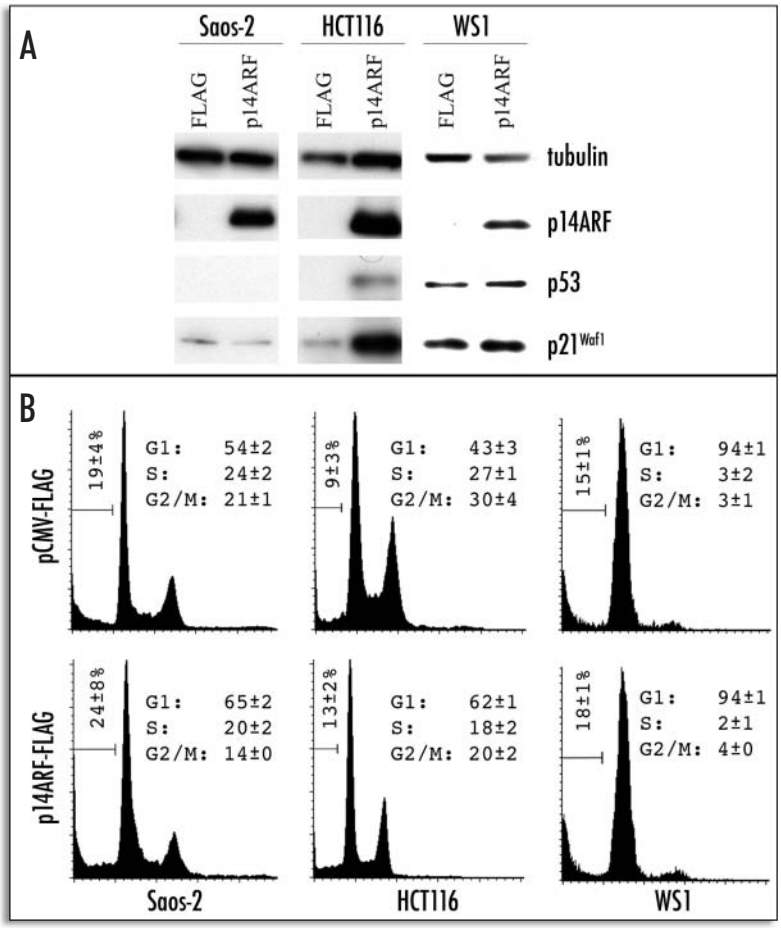

Figure 6. Transient p14ARF expression induces p53-dependent cell cycle arrest but no apoptosis in p53-intact or p53-deficient cells. Saos-2, HCT116 and WS1 fibroblast cells were transfected with the *p14ARF-FLAG* plasmid or *pCMV-FLAG* vector and *pCMVEGFP-spectrin*. (A) The expression of p14ARF-FLAG, p53, $p21^{Waf1}$ and tubulin was determined by Western blotting, 48 h post transfection. (B) The cell cycle distribution of green fluorescent cells was determined, 48 h post transfection, using propidium iodide staining. Each result is derived from at least two independent transfection experiments.

Table 1 **Effect of $p14^{ARF}$ expression on the drug sensitivity of cells**

Drug	p14ARF	Cells	% apoptosis	Ref.
25 μM DRB[a]	-	U20S_ARF	10 ± 1	this work
	+		29 ± 2	
50 μM DRB[a]	-	U20S_ARF	28 ± 2	this work
	+		49 ± 3	
1 μM adriamycin[b]	-	U20S_ARF	25 ± 1	this work
	+		37 ± 3	
1 μM CPT[b]	-	U20S_ARF	44 ± 5	this work
	+		50 ± 1	
10 μM CPT[a]	-	U20S_ARF	23 ± 4	this work
	+		26 ± 4	
5 μM cisplatin[c]	-	MCF7	~30	50
	+		~80	
10 U/ml IFNβ[d]	-	U20S_ARF	~20	56
	+		~40	

[a]U20S_ARF cells were treated with the indicated drug in the presence (+) of absence (-) of 1 mM IPTG for 3 days, and scored for apoptosis using FACS analysis. [b]U20S_ARF cells were treated with the indicated drug in the presence of absence of 1 mM IPTG for 6 h. The media was then removed and cells incubated for a further 72 h in media containing either PBS (-) or 1 mM IPTG (+) and scored for apoptosis using FACS analysis. [c]MCF7 cells were treated with cisplatin for 4 h and infected with adenovirus containing p14ARF (+) or control adenovirus (-) at 50 pfu/cell for 5 days. The proportion of apoptotic cells was retrieved from data showing relative survival.[50] [d]U20S cells stably expressing IPTG-inducible p14ARF were treated with IFNβ in the presence (+) of absence (-) of 1 mM IPTG for 3 days, and scored for apoptosis using Hoechst staining. Data recorded in this table was retrieved from Sandoval et al.[56]

p53 and $p21^{Waf1}$, but did not induce p14ARF expression (Fig. 7A). In the presence of IPTG, treatment with adriamycin consistently led to the accumulation of higher levels of p14ARF, with no further increases in the level of p53, compared with the adriamycin only or IPTG only treated cells. Nevertheless, the combination of p14ARF (IPTG treatment) and adriamycin treatment induced significantly higher levels of apoptosis than adriamycin treatment alone in U20S_ARF cells. In particular, the sub-G_1 population increased from 25% to 37% (Fig. 7B) and the proportion of Annexin-V positive, apoptotic cells increased from 22% to 30% (Fig. 7C). p14ARF induction also sensitized U20S_ARF cells to apoptosis in response to treatment with 25 μM or 50 μM DRB but not to short- (6 h) or long-term (72 h) treatment with camptothecin (Table 1).

DISCUSSION

Defining the tumour suppressor functions of human p14ARF has been complicated not only because this protein shares its genomic sequence with the *$p16^{INK4a}$* melanoma-predisposition gene, but also because much of the work on ARF has been performed on murine homologue. Murine and human ARF share only 49% amino acid homology, are differentially regulated (only p19ARF is induced by replicative senescence[55]), and interact different partners (only p19ARF binds pex19p[37]), Furthermore, the C-terminal region of the murine ARF protein is important for p19ARF-mediated apoptosis, but this region is not conserved in human p14ARF.[47] One of the critical functional differences between the mouse and human ARF proteins may be their role in promoting apoptosis; whereas p19ARF alone can promote cell death, the impact of p14ARF on apoptosis was not clearly defined. In this report, we have established that expression of p14ARF alone induces potent p53-dependent cell cycle arrest, but does not promote cell death in p53-null or p53-intact cells.

Although our data is supported by several reports[21,26,27] others have detected significant p14ARF-induced apoptosis. In these studies adenoviral-mediated p14ARF expression induced dose-dependent cell death. In particular, the strong overexpression of p14ARF was capable of inducing apoptosis, whereas lower but easily detectable expression levels of p14ARF did not induce significant death.[50,52,53] It also appears that long periods of ectopic ARF expression are necessary to induce apoptosis. In two reports, both using ectopic adenoviral-mediated p14ARF expression in HCT116 cells, apoptosis was detected at 72 h,[53] but not 24 h post infection (even though p14ARF induced cell cycle arrest at this earlier time point).[21] Perhaps the most revealing evidence regarding the apoptotic function of

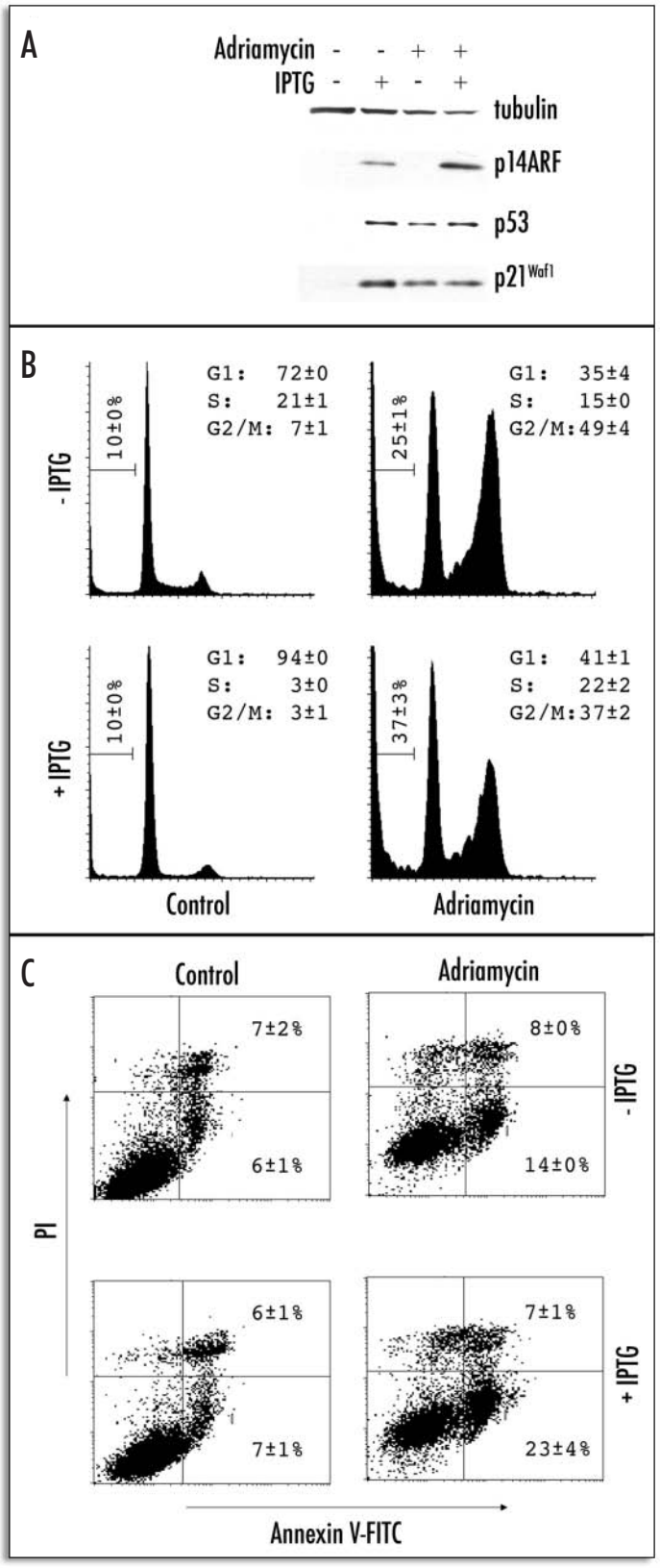

Figure 7. p14ARF sensitizes U20S cells to apoptosis. U20S_ARF cells were incubated with media containing either 1μM adriamycin and/or 1mM IPTG for 6 h. The adriamycin-containing media was then removed and cells incubated for a further 72 h in media containing either PBS (-) or 1mM IPTG (+), as indicated. (A) The expression of p14ARF, p53, $p21^{Waf1}$ and tubulin was determined by Western blotting. (B) The cell cycle distribution of propidium iodide stained nuclei was examined by flow cytometry. (C) Apoptosis was measured using annexin V staining. PI, propidium iodide.

p14ARF comes from studies indicating that additional cellular stresses, such as cisplatin-induced DNA damage or exposure to interferons in combination with ARF induction promoted apoptosis.[50,56] We have also confirmed that although p14ARF alone did not promote apoptosis in the p53-intact U20S cells, it did sensitize these cells to apoptosis following treatment with adriamycin and DRB, but not camptothecin. Similarly, whereas the expression of p14ARF in human breast cancer cells rendered them more susceptible to killing by cisplatin, a similar cooperative effect was not observed with the chemotherapeutic agent, Taxol.[50] The differential apoptotic response of p14ARF expressing cells following exposure to drugs requires further investigation, but anecdotal evidence suggests that it may involve p53. Specifically, both adriamycin and DRB can stimulate the p53 pathway; adriamycin promotes the nuclear accumulation and phosphorylation of p53[57,58] and DRB induces p53 by downregulation of mdm2.[59] In contrast, camptothecin- and Taxol-induced apoptosis can be p53-independent,[60,61] and in the p53-null 1175_ARF cells, p14ARF expression did not sensitize to apoptosis following exposure to adriamycin (data not shown). Regardless of the precise mechanism, it is clear that certain types of cellular stress in combination with p14ARF accumulation promote apoptosis. This may help explain previous reports showing that adenoviral-mediated p14ARF expression induces apoptosis.[50,52,53] Adenoviral gene delivery methods independently induce cellular stress[62] that may synergize with ectopic p14ARF expression to induce apoptosis. The stable cell models we have used in this study require very low levels of the nontoxic IPTG to induce accumulation of p14ARF, and thus provide a more physiological model to analyse p14ARF functions.

These data indicate that the primary response of cells to p14ARF accumulation is a potent and rapid p53-dependent cell cycle arrest. This is consistent with the senescent-like growth arrest induced in primary human cells by oncogenic stimuli that induce p14ARF, including RAS and E2F-1.[63] Activation of p14ARF alone does not promote apoptosis, but sensitizes cells to the pro-apoptotic effects of additional cellular stresses, including DNA damage. This may explain the importance of p14ARF in the development of melanoma. The majority of melanomas express a constitutively activated MAPK signalling pathway (principally via mutated BRAF), and although oncogenic BRAF is not sufficient to promote tumourigenesis,[64] it may sensitize melanocytes to apoptotic signals via the induction of p14ARF[65] and $p16^{INK4a}$.[66] The additional loss of p14ARF and $p16^{INK4a}$ in these tumors would remove this safeguard, and lead to MAPK proliferative signalling. Consistent with this model, recent analyses of primary and metastatic melanomas found that the most represented tumour genotype (17 of 41) was activated BRAF in combination with $p16^{INK4a}$ and p14ARF loss.[67]

References

1. Sherr CJ. Cancer cell cycles. Science 1996; 274:1672-7.
2. Kefford RF, Mann GJ. Is there a role for genetic testing in patients with melanoma? Curr Opin Oncol 2003; 15:157-61.
3. Soufir N, Avril M-F, Chompret A, Demenais F, Bombled J, Spatz A, Stoppa-Lyonnet D, The French Familial Melanoma Group, Bénard J, Bressac-de Paillerets B. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. Hum Mol Genet 1998; 7:209-16.
4. Holland EA, Schmid H, Kefford RF, Mann GJ. CDKN2A ($p16^{INK4a}$) and CDK4 mutation analysis in 131 Australian melanoma probands: Effect of family history and multiple primary melanomas. Genes Chromosomes Cancer 1999; 25:339-48.
5. Walker GJ, Hussussian CJ, Flores JF, Glendening JM, Haluska FG, Dracopoli NC, Hayward NK, Fountain JW. Mutations of the *CDKN2/p16^{INK4}* gene in Australian melanoma kindreds. Hum Mol Gen 1995; 4:1845-52.
6. Pollock PM, Pearson JV, Hayward NK. Compilation of somatic mutations of the *CDKN2* gene in human cancers: Nonrandom distribution of base substitutions. Genes Chromosomes Cancer 1996; 15:77-88.
7. Rizos H, Puig S, Badenas C, Malvehy J, Darmanian AP, Jiménez L, Milà M, Kefford RF. A melanoma-associated germline mutation in exon 1β inactivates p14ARF. Oncogene 2001; 20:5543-7.
8. Randerson-Moor JA, Harland M, Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J, Sibley K, Whitaker L, Knowles M, Newton Bishop J, Bishop DT. A germline deletion of $p14^{ARF}$ but not CDKN2A in a melanoma-neural system tumour syndrome family. Hum Mol Genet 2001; 10:55-62.
9. Hewitt C, Wu CL, Evan G, Howell A, Elles RG, Jordan R, Sloan P, Read AP, Thakker N. Germline mutation of ARF in a melanoma kindred. Hum Mol Genet 2002; 11:1273-9.
10. Freedberg DE, Mistry S, Russak J, Gai W, Kaplow M, Osman I, Turner F, Houghton A, Busam KJ, Bishop T, Bastian BC, Newton Bishop JA, Polsky D. Genetic and epigenetic alterations of p14ARF are frequent in human melanoma metastases. Phoenix, Arizona, USA: Second Melanoma Research Congress, 2004.
11. Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 1998; 92:725-34.
12. Pomerantz J, Schreiber-Agus N, Liégeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee H-W, Cordon-Cardo C, DePinho RA. The Ink4a tumor suppressor gene product, $p19^{Arf}$, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 1998; 92:713-23.
13. Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF, Sherr CJ. Functional and physical interactions of the ARF tumor suppressor with p53 and mdm2. Proc Natl Acad Sci USA 1998; 95:8292-7.
14. Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, Palmero I, Ryan K, Hara E, Vousden KH, Peters G. The alternative product from the human CDKN2A locus, $p14^{ARF}$, participates in a regulatory feedback loop with p53 and MDM2. EMBO J 1998; 17:5001-14.
15. Palmero I, Pantoja C, Serrano M. $p19^{ARF}$ links the tumour suppressor p53 to Ras. Nature 1998; 395:125-6.
16. Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev 1998; 12:2424-33.
17. Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, Vousden KH. p14ARF links the tumour suppressors RB and p53. Nature 1998; 395:124-5.
18. Sherr CJ. An Arf(GFP/GFP) reporter mouse reveals that the Arf tumor suppressor monitors latent oncogenic signals in vivo. Cell Cycle 2004; 3:239-40.
19. Khan SH, Moritsugu J, Wahl GM. Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion. Proc Natl Acad Sci USA 2000; 97:3266-71.
20. Khan S, Guevara C, Fujii G, Parry D. p14ARF is a component of the p53 response following ionizing radiation of normal human fibroblasts. Oncogene 2004; 23:6040-6.
21. Weber HO, Samuel T, Rauch P, Funk JO. Human p14ARF-mediated cell cycle arrest strictly depends on intact p53 signaling pathways. Oncogene 2002; 21:3207-12.
22. Carnero A, Hudson JD, Price CM, Beach DH. $p16^{INK4a}$ and p19ARF act in overlapping pathways in cellular immortalization. Nat Cell Biol 2000; 2:148-55.
23. Weber JD, Jeffers JR, Rehg JE, Randle DH, Lozano G, Roussel MF, Sherr CJ, Zambetti GP. p53-independent functions of the p19ARF tumor suppressor. Genes Dev 2000; 14:2358-65.
24. Esteller M, et al. Hypermethylation-associated inactivation of p14ARF is independent of p16INK4a methylation and p53 mutational status. Cancer Res 2000; 60:129-33.
25. Sanchez-Cespedes M, Reed AL, Buta M, Wu L, Westra WH, Herman JG, Yang SC, Jen J, Sidransky D. Inactivation of the INK4A/ARF locus frequently coexists with TP53 mutations in nonsmall cell lung cancer. Oncogene 1999; 18:5843-9.
26. Yarbrough WG, Bessho M, Zanation A, Bisi JE, Xiong Y. Human tumour suppressor ARF impedes S-phase progression independent of p53. Cancer Res 2002; 62:1171-7.
27. Eymin B, Karayan L, Séité P, Brambilla C, Brambilla E, Larson C-J, Gazzéri S. Human ARF binds E2F1 and inhibits its transcriptional activity. Oncogene 2001; 20:1033-41.
28. Normand G, Hemmati PG, Verdoodt B, von Haefen C, Wendt J, Guner D, May E, Dorken B, Daniel PT. p14ARF induces G_2 cell cycle arrest in p53 and p21 deficient cells by downregulating p34cdc2 kinase activity. J Biol Chem 2004; 6:6.
29. Sugimoto M, Kuo M-L, Roussel MF, Sherr CJ. Nucleolar Arf tumor suppressor inhibits ribosomal RNA processing. Mol Cell 2003; 11:415-24.
30. Mason SL, Loughran O, La Thangue NB. p14ARF regulates E2F activity. Oncogene 2002; 21:4220-30.
31. Datta A, Nag A, Raychaudhuri P. Differential regulation of E2F1, DP1 and the E2F1/DP1 complex by ARF. Mol Cell Biol 2002; 22:8398-408.
32. Vivo M, Calogero RA, Sansone F, Calabrò V, Parisi T, Borrelli L, Saviozzi S, La Mantia G. The human tumor suppressor ARF interacts with spinophilin/neurabin II, a type 1 protein-phosphatase-binding protein. J Biol Chem 2001; 276:14161-9.
33. Karayan L, Riou J-F, Séité P, Migeon J, Cantereau A, Larson C-J. Human ARF protein interacts with Topoisomerase I and stimulates its activity. Oncogene 2001; 20:836-48.
34. Zhao L, Samuels T, Winckler S, Korgaonkar C, Tompkins V, Horne MC, Quelle DE. Cyclin G_1 has growth inhibitory activity linked to the ARF-Mdm2-p53 and pRb tumor suppressor pathways. Mol Cancer Res 2003; 1:195-206.
35. Jackson MW, Lindström MS, Berberich SJ. MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. Oncogene 2001; 276:25336-41.
36. Fatyol K, Szalay AA. The $p14^{ARF}$ tumour suppressor protein facilitates nucleolar sequestration of hypoxia-inducible factor 1a (HIF-1a) and inihibits HIF-1 mediated transcription. J Biol Chem 2001; 276:28421-9.
37. Sugihara T, Kaul SC, Kato J, Reddel RR, Nomura H, Wadhwa R. Pex19p dampens the p19ARF-p53-p21WAF1 tumor suppressor pathway. J Biol Chem 2001; 276:18649-52.
38. Hasan MK, Yaguchi T, Sugihara T, Kumar PKR, Taira K, Reddel RR, Kaul SC, Wadhwa R. CARF is a novel protein that cooperates with mouse $p19^{ARF}$ (human $p14^{ARF}$) in activating p53. J Biol Chem 2002; 277:37765-70.
39. Rizos H, Diefenbach E, Badhwar P, Woodruff S, Becker TM, Rooney RJ, Kefford RF. Association of p14ARF with the $p120^{E4F}$ transcriptional repressor enhances cell cycle inhibition. J Biol Chem 2003; 278:4981-9.
40. Itahana K, Bhat KP, Jin A, Itahana Y, Hawke D, Kobayashi R, Zhang Y. Tumor Suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation. Mol Cell 2003; 12:1151-61.
41. Woods YL, Xirodimas DP, Prescott AR, Sparks A, Lane DP, Saville MK. p14Arf promotes SUMO conjugation of Werners helicase. J Biol Chem 2004; 279:50157-66.
42. Martelli F, Hamilton T, Silver DP, Sharpless NE, Bardeesy N, Rokas M, DePinho RA, Livingston DM, Grossman SR. p19ARF targets certain E2F species for degradation. Proc Natl Acad Sci USA 2001; 98:4455-60.
43. Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell 1995; 83:993-1000.
44. de Stanchina E, McCurrach ME, Zindy F, Shieh S-Y, Ferbeyre G, Samuelson AV, Prives C, Roussel MF, Sherr CJ, Lowe SW. E1A signaling to p53 involves the $p19^{ARF}$ tumor suppressor. Genes Dev 1998; 12:2434-42.
45. Russell JL, Poers JT, Rounbehler RJ, Rogers PM, Conti CJ, Johnson DG. ARF differentially modulates apoptosis induced by E2F1 and Myc. Mol Cell Biol 2002; 22:1360-1368.
46. Tsuji K, Mizumoto K, Sudo H, Kouyama K, Ogata E, Matsuoka M. p53-independent apoptosis is induced by the p19ARF tumor suppressor. Biochem Biophys Res Commun 2002; 295:621-9.
47. Matsuoka M, Kurita M, Sudo H, Mizumoto K, Nishimoto I, Ogata E. Multiple domains of the mouse $p19^{ARF}$ tumor suppressor are involved in p53-independent apoptosis. Biochem Biophys Res Commun 2003; 301:1000-10.
48. Drayton S, Rowe J, Jones R, Vatcheva R, Cuthbert-Heavens D, Marshall J, Fried M, Peters G. Tumor suppressor p16INK4a determines sensitivity of human cells to transformation by cooperating cellular oncogenes. Cancer Cell 2003; 4:301-10.
49. Eymin B, Leduc C, Coll JL, Brambilla E, Gazzeri S. $p14^{ARF}$ induces G_2 arrest and apoptosis independently of p53 leading to regression of tumours established in nude mice. Oncogene 2003; 22:1822-35.
50. Deng Q, Kim H, Vandier D, Jung Y, Rikiyama T, Sgagias MK, Goldsmith M, Cowan KH. Recombinant adenovirus-mediated p14ARF overexpression sensitizes human breast cancer cells to cisplatin. Biocem Biophys Res Commun 2002; 296:792-8.
51. Yang CT, You L, Yeh, CC, Chang JW, Zhang F, McCormick F, Jablons DM. Adenovirus-mediated *p14ARF* gene transfer in human mesothelioma cells. J Natl Cancer Inst 2000; 92:636-41.
52. Kim M, Sgagias M, Deng X, Jung YJ, Rikiyama T, Lee K, Ouellette M, Cowan K. Apoptosis induced by adenovirus-mediated p14ARF expression in U2OS osteosarcoma cells is associated with increased Fas expression. Biochem Biophys Res Commun 2004; 320:138-44.
53. Hemmati PG, Gillissen B, von Haefen C, Wendt J, Starck L, Güner D, Dorken B, Daniel PT. Adenovirus-mediated overexpression of p14ARF induces p53 and Bax-independent apoptosis. Oncogene 2002; 21:3149-3161.
54. Rizos H, Darmanian AP, Indsto JO, Shannon JA, Kefford RF, Mann GJ. Multiple abnormalities of the $p16^{INK4a}$-pRb regulatory pathway in cultured melanoma cells. Melanoma Res 1999; 9:10-9.
55. Brookes S, Rowe J, Ruas M, Llanos S, Clark PA, Lomax M, James MC, Vatcheva R, Bates S, Vousden KH, Parry D, Gruis N, Smit N, Bergman W, Peters G. INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. EMBO J 2002; 21:2936-45.
56. Sandoval R, Xue J, Pilkinton M, Salvi D, Kiyokawa H, Colamonici OR. Different requirements for the cytostatic and apoptotic effects of type I IFNs: Induction of apoptosis requires ARF, but not p53 in osteosarcoma cell lines. J Biol Chem 2004; 279:32275-80.

57. Fritsche M, Haessler C, Brandner G. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene 1993; 8:307-18.
58. Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E, Anderson CW. ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. J Biol Chem 2002; 277:12491-4.
59. Blagosklonny MV, Demidenko ZN, Fojo T. Inhibition of transcription results in accumulation of Wt p53 followed by delayed outburst of p53-inducible proteins: p53 as a sensor of transcriptional integrity. Cell Cycle 2002; 1:67-74.
60. Bacus SS, Gudkov AV, Lowe M, Lyass L, Yung Y, Komarov AP, Keyomarsi K, Yarden Y, Seger R. Taxol-induced apoptosis depends on MAP kinase pathways (ERK and p38) and is independent of p53. Oncogene 2001; 20:147-55.
61. Paquet C, Schmitt E, Beauchemin M, Bertrand R. Activation of multidomain and BH3-only pro-apoptotic Bcl-2 family members in p53-defective cells. Apoptosis 2004; 9:815-31.
62. Brand K, Klocke R, Possling A, Paul D, Strauss M. Induction of apoptosis and G_2/M arrest by infection with replication-deficient adenovirus at high multiplicity of infection. Gene Ther 1999; 6:1054-63.
63. Berkovich E, Lamed Y, Ginsberg D. E2F and Ras synergize in transcriptionally activating p14ARF expression. Cell Cycle 2003; 2:127-33.
64. Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, Moses TY, Hostetter G, Wagner U, Kakareka J, Salem G, Pohida T, Heenan P, Duray P, Kallioniemi O, Hayward NK, Trent JM, Meltzer PS. High frequency of BRAF mutations in nevi. Nat Genet 2003; 33:19-20.
65. Groth A, Weber JD, Willumsen BM, Sherr CJ, Roussel MF. Oncogenic Ras induces p19ARF and growth arrest in mouse embryo fibroblasts lacking p21Cip1 and p27Kip1 without activating cyclin D-dependent kinases. J Biol Chem 2000; 275:27473-80.
66. Lin AW, Barradas M, Stone JC, van Aelst L, Serrano M, Lowe SW. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev 1998; 12:3008-19.
67. Daniotti M, Oggionni M, Ranzani T, Vallacchi V, Campi V, Di Stasi D, Torre GD, Perrone F, Luoni C, Suardi S, Frattini M, Pilotti S, Anichini A, Tragni G, Parmiani G, Pierotti MA, Rodolfo M. BRAF alterations are associated with complex mutational profiles in malignant melanoma. Oncogene 2004; 23:5968-77.