p19\textsuperscript{Arf} induces p53-dependent apoptosis during Abelson virus-mediated pre-B cell transformation

ARASH RADFAR\textsuperscript{*†}, INDIRA UNNIKRISHNAN\textsuperscript{‡}, HAN-WOONG LEE\textsuperscript{§}, RONALD A. DEPINHO\textsuperscript{¶}, AND NAOMI ROSENBERG\textsuperscript{*†‡}

ABSTRACT  The \textit{Ink4a/Arf} locus encodes p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} and is among the most frequently mutated tumor suppressor loci in human cancer. In mice, many of these effects appear to be mediated by interactions between p19\textsuperscript{Arf} and the p53 tumor-suppressor protein. Because \textit{Tp53} mutations are a common feature of the multistep pre-B cell transformation process mediated by Abelson murine leukemia virus (Ab-MLV), we examined the possibility that proteins encoded by the \textit{Ink4a/Arf} locus also play a role in Abelson virus transformation. Analyses of primary transformants revealed that both p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} are expressed in many of the cells as they emerge from the apoptotic crisis that characterizes the transformation process. Analyses of primary transformants from \textit{Ink4a/Arf} null mice revealed that these cells bypassed crisis. Because expression of p19\textsuperscript{Arf} but not p16\textsuperscript{Ink4a} induced apoptosis in Ab-MLV-transformed pre-B cells, p19\textsuperscript{Arf} appears to be responsible for these events. Consistent with the link between p19\textsuperscript{Arf} and p53, \textit{Ink4a/Arf} expression correlates with or precedes the emergence of cells expressing mutant p53. These data demonstrate that p19\textsuperscript{Arf} is an important part of the cellular defense mounted against transforming signals from the Abl oncprotein and provide direct evidence that the p19\textsuperscript{Arf}–p53 regulatory loop plays an important role in lymphoma induction.

Tumor induction is a multistep process involving activation of oncogenes and inactivation of tumor-suppressor genes (1, 2). One experimental system to which this paradigm applies is transformation by Abelson murine leukemia virus (Ab-MLV), an oncogenic retrovirus that encodes the v-Ab1 protein tyrosine kinase (3). The virus induces mono- or oligoclonal pre-B cell tumors (4, 5), and clonal selection also occurs in vitro. Primary pre-B cell transformants are poorly oncogenic and undergo an apoptotic crisis soon after isolation (6–9). Cells that survive are rapidly growing and highly tumorigenic (6–8). Mutations in the \textit{Tp53} tumor-suppressor gene become evident in about half of the surviving clones as they emerge from crisis or soon thereafter (8), and primary Ab-MLV transformants derived from \textit{Tp53}−/− mice bypass crisis and become established rapidly (I.U., A.R., and N.R., unpublished data). Thus, p53 is one important cellular defense against malignant transformation by Ab-MLV.

p53 is regulated by multiple factors, including p19\textsuperscript{Arf}, a product of the \textit{Ink4a/Arf} locus (10–15). p19\textsuperscript{Arf} is part of the protective cellular response to oncogenic insults from at least two oncogenes, c-Myc and adenovirus \textit{E1A} (13, 14). p19\textsuperscript{Arf} stabilizes and activates p53, probably by forming a complex with p53 and the Mdm2 protein (11, 12, 15), a molecule that normally increases p53 ubiquitination and degradation (16, 17). Signals from p19\textsuperscript{Arf} to p53 induce G1 arrest and apoptosis in fibroblast cells and are critical for the normal senescence response (10, 13, 14, 18). Consistent with this mechanism and the importance of p53 in tumorigenesis (reviewed in refs. 19 and 20), mice lacking a functional p19\textsuperscript{Arf} gene develop multiple types of tumors at a high frequency (18).

Although murine models strongly support the idea that p19\textsuperscript{Arf} is a tumor-suppressor protein, evidence for its role in naturally occurring tumors is limited. Indeed, in this arena, attention has focused on the second product of the \textit{Ink4a/Arf} locus, the p16\textsuperscript{Ink4a} cyclin-dependent kinase inhibitor (21). This protein inhibits the activity of CDK-4 and CDK-6, suppressing their ability to phosphorylate pRb, an event that induces G1 arrest (21–24). p16\textsuperscript{Ink4a} mutations or altered p16\textsuperscript{Ink4a} expression have been documented in many types of tumors, including leukemias and lymphomas (reviewed in ref. 25). Although some of these changes affect both p19\textsuperscript{Arf} and p16\textsuperscript{Ink4a}, others affect only p16\textsuperscript{Ink4a} (26–30).

We investigated the role of p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} in Ab-MLV induced pre-B cell transformation to determine whether selection for \textit{Tp53} mutation involves the p19\textsuperscript{Arf}–p53 regulatory loop or whether altered expression of p16\textsuperscript{Ink4a} occurs in transformants retaining wt p53. Our studies reveal that primary transformants derived from \textit{Ink4a/Arf}−/− mice do not undergo crisis. Because overexpression of p19\textsuperscript{Arf}, but not p16\textsuperscript{Ink4a}, induces apoptosis in a p53-dependent fashion in the transformants, these effects appear to be mediated by p19\textsuperscript{Arf}. These data identify a tumor-suppressor function for p19\textsuperscript{Arf} in Ab-MLV-induced pre-B cell transformation. Furthermore, because elevated expression of p19\textsuperscript{Arf} can precede emergence of cells harboring \textit{Tp53} mutations, high levels of p19\textsuperscript{Arf} appear to select for cells expressing mutant forms of p53.

MATERIALS AND METHODS

Cells and Mice. Ab-MLV-transformed pre-B cell lines and NIH 3T3 cells were grown as described previously (8, 31). The pre-B cell transformants 379-10, 143-2M, and 300-31 express mutant p53, which fails to induce apoptosis after DNA damage and does not activate expression from a promoter containing p53-responsive elements (8); 298-18, 204-3-1, and 38B9 express wild-type (wt) p53 (8). L1-2 is a p53 null cell line (32). 379-10, 298-18, and 38B9 are transformed with Ab-MLV-P160; 143-2M, 300-31, 204-3-1, and L1-2 are transformed with Ab-MLV-P120. Both of these Ab-MLV strains are considered wild type (3). MEL cells are murine erythroleukemic cells that express p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} (33, 34). For isolating primary transformants, bone marrow cells were infected with Ab-MLV-P160 and plated in soft agar (9). Primary transformants were plated in 24-well plates 10 days later in supplemented RPMI medium.

Abbreviations: Ab-MLV, Abelson murine leukemia virus; MC540, merocyanin 540; GFP, green fluorescent protein; wt, wild type.

**To whom reprint requests should be addressed: SC315, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. e-mail: nrosenbe@opal.tufts.edu.

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(RPMMi 1640 medium/2 mM l-glutamine/50 μM 2-mercapto-ethanol) containing 20% fetal calf serum. When the cells filled the wells, half of them were transferred to a new well. When the viability exceeded 80%, the cells were transferred to 35-mm dishes and subcultured as before. When the cells consistently maintained a viability of >90% and could be subcultured on regular schedule, they were considered established. In some experiments, pre-B transformants were treated with approximately 1,000-rad γ-irradiation (8); cell viability was scored the following day either by visual inspection, trypan blue staining, or mercocyanin 540 (MC540, Molecular Probes) staining. BALB/cByJ and mixed background cByJ and mixed background cByJ and mixed background cByJ and mixed background mice (35) were bred at Tufts University.

**RNA and Protein Analysis.** Total RNA was prepared by using the RNeasy Mini Kit (Qiagen) and analyzed by Northern blotting (36, 37). The p16\(^{ink4a}\) exon 1 probe was prepared by PCR amplification of bases 6–198 of the p16 cDNA (33); the p19\(^{Arf}\) exon 1 probe was prepared by PCR amplification of bases 14–229 of p19 Arf cDNA (10). Cell lysates for protein analyses were prepared and analyzed by Western blotting (31) by using anti-p16\(^{ink4a}\) antibodies, anti-CDK-4, anti-CDK-6, and anti-cyclin D3 (M-156, C-22, C-21, and C-16, respectively, Santa Cruz Biotechnology), anti-p19\(^{Arf}\) (res. 11; R.A.D., unpublished data), and anti-Gag/v-Ab antibodies (HS458, 38) antibodies.

**Transfections.** To construct p16\(^{ink4a}\) and p19\(^{Arf}\) expression plasmids, the EcorI fragment containing the full-length p16\(^{ink4a}\) cDNA from pKS-mp16 (33) or the ClaI fragment containing the full-length p19\(^{Arf}\) cDNA and the codons for the hemagglutinin tag from a pSR-antiv-Abl plasmid (38) were inserted into the pCI vector (Promega). Before transfection, the cells were washed twice in unsupplemented RPMI 1640 and mixed with the p16\(^{ink4a}\) or p19\(^{Arf}\) expression plasmids and pCMVEGFP-Spectrin (39) at a ratio of 5:1, pCMVEGFP-Spectrin encodes green fluorescent protein (GFP) fused to the transmembrane domain of spectrin (39). The cells were incubated for 10 min at room temperature and electroporated at 300 V. The cells were incubated on ice for 10 min, plated in supplemented RPMI containing 10% fetal calf serum, and incubated for 24 hr. The cells were harvested, washed twice with Mg\(^{2+}\)- and Ca\(^{2+}\)-free PBS supplemented with 0.1% BSA, mixed with MC540 at a final concentration of 0.25 μg/ml, and analyzed immediately by flow cytometry. Apoptotic cells are specifically stained with MC540 (40). Gates were set on the GFP-positive cells, and this population was analyzed for live and apoptotic cells.

**RESULTS**

**p16\(^{ink4a}\) and p19\(^{Arf}\) Are Expressed in Transformants Exposing Mutant Tp53.** To determine whether a correlation between expression of the Ink4a/Arf locus and Tp53 status exists in Ab-MLV transformed pre-B cells, their expression was examined in a panel of transformants with known Tp53 status (8). Northern analysis revealed that a p53-null cell line (32) and two of the three cell lines expressing mutant p53 expressed readily detectable levels of both RNAs (Fig. 1A). Both RNAs could be detected in samples from Mut3 on prolonged exposure at levels about 10- to 20-fold less than those in Mut1. None of the cell lines expressing wt p53 expressed high levels of both p16\(^{ink4a}\) and p19\(^{Arf}\). Wt2 expressed abundant p16\(^{ink4a}\) RNA, but p19\(^{Arf}\) RNA was detectable only on prolonged exposure at about 50-fold-lower levels. Wt1 lacked detectable p16\(^{ink4a}\) RNA, but prolonged exposures revealed about 50-fold-less p19\(^{Arf}\) RNA than found in strongly positive samples; Wt3 expressed about 10-fold-less p16\(^{ink4a}\) RNA than the highly positive samples but lacked detectable p19\(^{Arf}\) RNA. Western analyses revealed that cells expressing abundant Ink4a/Arf locus RNAs expressed readily detectable levels of the corresponding proteins (Fig. 1B). Consistent with a relationship between Ink4a/Arf locus expression and p53 status, three of four other cell lines expressing mutant p53 expressed both p16\(^{ink4a}\) and p19\(^{Arf}\). Only 1 of 18 cell lines with wt p53 expressed high levels of p19\(^{Arf}\); 4 expressed high levels of p16\(^{ink4a}\) and none expressed high levels of both proteins (data not shown).

**p16 and p19\(^{Arf}\) Can Be Up-Regulated Before Tp53 Mutations Arise.** Mutation of Tp53 is usually a late, postcrisis event in Ab-MLV transformation (8). Because the loss of p53 that often accompanies fibroblast cell immortalization is preceded by changes in Ink4a/Arf locus expression (14, 18, 35), we examined primary transformants for expression of Ink4a/Arf locus products and acquisition of Tp53 mutations. None of 10 primary transformants, including the representatives shown (Fig. 2A), expressed detectable levels of p16\(^{ink4a}\) or p19\(^{Arf}\) when they were isolated from agar. The primary transformants were cultured, and, as expected (8), crisis began within 2–5 days and usually lasted for 25–35 days. Samples were collected every 10–21 days as the health of the cells allowed. Analyses of serial samples from the 13 primary transformants that became established revealed that 8 had become p16\(^{ink4a}/\)p19\(^{Arf}\)-positive as they emerged from crisis; 1 primary transformant expressed only p16\(^{ink4a}\). In the representative examples shown (Fig. 2B), primary transformants 1, 2, and 4 were considered established at day 35, 40, and 33, respectively;
primary transfectants from BALB/c mice and Ink4a/Arf−/− mice (35) was compared. In both cases, the primary transfectants contained >90% viable cells when they were explanted from agar. As expected based on analyses of primary transfectants from several strains of inbred mice (I.U., A.R., and N.R., unpublished data and our unpublished data), a high percentage of the cells in all 48 primary transfectants from wild-type mice underwent apoptotic crisis beginning 2–5 days after explant and continuing for about a month. MCS50 staining of cells from cultures containing viable cells revealed that 50–80% of the cells showed evidence of apoptosis (Fig. 4); 15 of 48 primary transfectants survived crisis to become established while all of the cells in the other 33 died by apoptosis during the crisis period (representatives are shown in Fig. 5). In contrast, none of the 48 primary transfectants from Ink4a/Arf−/− mice underwent extensive cell death as judged by visual inspection; MCS50 staining indicated that fewer than 30% of the cells in these samples showed evidence of apoptosis (Fig. 4). These cells grew exponentially, becoming fully established in less than 10 days (representatives are shown in Fig. 5). Although the genetic background of the control and Ink4a−/− mice used here is not identical, results similar to those obtained with BALB/c cells have been observed in seven other inbred strains and four strains of mixed background similar to that of the Ink4a−/− mice (our unpublished data). Thus, expression of Ink4a/Arf locus products is required for the apoptotic crisis that characterizes Ab-MLV-induced pre-B cell transformation.

Expression of p19Arf Induces p53-Dependent Apoptosis in Transformed Pre-B Cells. The Ink4a/Arf−/− mice used in our experiments express neither p16Ink4a nor p19Arf (35). To determine the effect of each protein on Ab-MLV-transformed pre-B cells, vectors expressing them were introduced into cells with known p53 status. The plasmids were mixed at a 5-fold excess with a plasmid encoding GFP-spectrin (39) and introduced into the cells by electroporation. GFP-spectrin is re-

**Fig. 2.** p16Ink4a and p19Arf up-regulation in primary transfectants. (A) Primary transfectants (Primary Tx) were analyzed for the expression of p16Ink4a and p19Arf by Western blotting 24 hr after explant from agar. The blots also were probed with an anti-CDK-4 antibody to control for protein loading. Lane +, lysate from NIH 3T3 cells; lane −, lysate from NIH 3T3 cells. (B) Western blots of lysates from four independent primary transfectants harvested at different days post-explant were probed with anti-p16Ink4a and p19Arf antibodies. The samples shown are representative of 13 independent primary transfectants that became established. Probing with anti-CDK-6 (Primary Tx 1, 4) or anti-cyclin D3 (Primary Tx 2, 3) antibodies controlled for protein loading. d, days after explant from agar.

To determine whether any of these cells expressed mutant p53, the susceptibility of the cells to γ-irradiation-induced apoptosis was monitored as soon as the cells emerged from crisis. Our earlier work has shown a strong correlation between resistance to radiation-induced apoptosis and Tp53 mutation in Ab-MLV-transformed pre-B cells (8). By 60 days, all eight of the p16Ink4a/p19Arf-positive cell lines expressed mutant p53 as judged by resistance to irradiation-induced apoptosis; in contrast, all of the other cell lines, including one that expressed only p16Ink4a, underwent rapid apoptosis after irradiation (data not shown). Comparison of the times at which abundant expression of Ink4a/Arf locus products occurred and Tp53 mutations were detected revealed that p16Ink4a/p19Arf expression became evident 10–14 days before Tp53 mutation appeared in four instances (Fig. 3, samples 1–4). In four other cases, none of the available samples distinguished a temporal order for these changes (represented by sample 6, Fig. 3). However, there were no cases in which Tp53 mutation occurred before abundant expression of Ink4a/Arf locus products. These data suggest that Ink4a/Arf locus products play a key role in the crisis period that characterizes the transformation process and that their expression can precede and may select for mutation in Tp53.

**Primary Pre-B Cell Transfectants from Ink4a/Arf−/− Mice Bypass Crisis.** To test the hypothesis that Ink4a/Arf locus products play a role in crisis induction, the outgrowth of primary transfectant 3 never expressed Ink4a locus products but became established at day 38. To determine whether any of these cells expressed mutant p53, the susceptibility of the cells to γ-irradiation-induced apoptosis was monitored as soon as the cells emerged from crisis. Our earlier work has shown a strong correlation between resistance to radiation-induced apoptosis and Tp53 mutation in Ab-MLV-transformed pre-B cells (8). By 60 days, all eight of the p16Ink4a/p19Arf-positive cell lines expressed mutant p53 as judged by resistance to irradiation-induced apoptosis; in contrast, all of the other cell lines, including one that expressed only p16Ink4a, underwent rapid apoptosis after irradiation (data not shown). Comparison of the times at which abundant expression of Ink4a/Arf locus products occurred and Tp53 mutations were detected revealed that p16Ink4a/p19Arf expression became evident 10–14 days before Tp53 mutation appeared in four instances (Fig. 3, samples 1–4). In four other cases, none of the available samples distinguished a temporal order for these changes (represented by sample 6, Fig. 3). However, there were no cases in which Tp53 mutation occurred before abundant expression of Ink4a/Arf locus products. These data suggest that Ink4a/Arf locus products play a key role in the crisis period that characterizes the transformation process and that their expression can precede and may select for mutation in Tp53.

**Primary Pre-B Cell Transfectants from Ink4a/Arf−/− Mice Bypass Crisis.** To test the hypothesis that Ink4a/Arf locus products play a role in crisis induction, the outgrowth of primary transfectants from BALB/c mice and Ink4a/Arf−/− mice (35) was compared. In both cases, the primary transfectants contained >90% viable cells when they were explanted from agar. As expected based on analyses of primary transfectants from several strains of inbred mice (I.U., A.R., and N.R., unpublished data and our unpublished data), a high percentage of the cells in all 48 primary transfectants from wild-type mice underwent apoptotic crisis beginning 2–5 days after explant and continuing for about a month. MCS50 staining of cells from cultures containing viable cells revealed that 50–80% of the cells showed evidence of apoptosis (Fig. 4); 15 of 48 primary transfectants survived crisis to become established while all of the cells in the other 33 died by apoptosis during the crisis period (representatives are shown in Fig. 5). In contrast, none of the 48 primary transfectants from Ink4a/Arf−/− mice underwent extensive cell death as judged by visual inspection; MCS50 staining indicated that fewer than 30% of the cells in these samples showed evidence of apoptosis (Fig. 4). These cells grew exponentially, becoming fully established in less than 10 days (representatives are shown in Fig. 5). Although the genetic background of the control and Ink4a−/− mice used here is not identical, results similar to those obtained with BALB/c cells have been observed in seven other inbred strains and four strains of mixed background similar to that of the Ink4a−/− mice (our unpublished data). Thus, expression of Ink4a/Arf locus products is required for the apoptotic crisis that characterizes Ab-MLV-induced pre-B cell transformation.

Expression of p19Arf Induces p53-Dependent Apoptosis in Transformed Pre-B Cells. The Ink4a/Arf−/− mice used in our experiments express neither p16Ink4a nor p19Arf (35). To determine the effect of each protein on Ab-MLV-transformed pre-B cells, vectors expressing them were introduced into cells with known p53 status. The plasmids were mixed at a 5-fold excess with a plasmid encoding GFP-spectrin (39) and introduced into the cells by electroporation. GFP-spectrin is re-
tained in cells undergoing apoptosis because the spectrin portion anchors the molecule inside the cell (39). The cells were harvested 24 hr later, stained with MC540, and analyzed by flow cytometry with gates set on the GFP-positive cells to assess the percentage of apoptotic cells. Expression of either p16Ink4a or p19Arf in p53 null cells (Fig. 6) or cells expressing mutant p53 (not shown) did not affect significantly the percentage of apoptotic cells when these samples were compared with those that had received an equivalent amount of empty vector. However, expression of p19Arf in cells expressing wt p53 increased the percentage of apoptotic cells by about 50% compared with cells receiving empty vector (Fig. 6). The slight increase in apoptotic cells observed in cells receiving either the p16Ink4a or p19Arf vector was not reproducible. These results suggest that expression of p19Arf induces p53-dependent apoptosis in Ab-MLV-transformed pre-B cells and that p19Arf is the active Ink4a/Arf locus product responsible for crisis induction in primary Ab-MLV-transformed pre-B cells.

**DISCUSSION**

Our experiments show that the Ink4a/Arf locus plays a major role in Ab-MLV-mediated pre-B cell transformation and identify these genes as one component of the cellular defense mounted against v-Abl-mediated transformation. Unlike pre-B transformants derived from normal mice (6–9), those from Ink4a/Arf−/− mice bypass the crisis that characterizes the transition from primary transformant to established, fully malignant cell line. In addition, all of the primary transformants become established in a very short time. In these respects, the primary Ab-MLV transformants are similar to mouse embryo cells derived from the null animals; these fibroblasts do not senesce in vitro and give rise to a high frequency of established cell lines rapidly (10, 13, 14, 18, 35).

![Fig. 5. Primary pre-B cell transformants from Ink4a/Arf−/− mice are established rapidly.](https://example.com/fig5)

**Fig. 5.** Primary pre-B cell transformants from Ink4a/Arf−/− mice are established rapidly. The growth and viability of 10 representative primary pre-B transformants derived from Ink4a/Arf−/− and wild-type mice is illustrated. A total of 48 primary transformants from each strain were analyzed. For wild-type cells, 33 of 48 primary transformants succumbed to crisis between day 7 and day 24; 15 of 48 primary transformants became established between day 28 and day 39. For Ink4a−/− cells, all 48 primary transformants became established between days 9 and 11. Open circles, primary transformants; shaded circles, transformants that succumbed to crisis; solid circles, established cell lines.

![Fig. 6. p19Arf induces apoptosis in pre-B cell transformants expressing wild-type p53.](https://example.com/fig6)

**Fig. 6.** p19Arf induces apoptosis in pre-B cell transformants expressing wild-type p53. Pre-B cell transformants expressing wild-type p53 (Wt1, 204–3-1) and (Wt2, 38B9) and the p53 null transformant L1–2 (8, 32) were electroporated with pCMVEGFP-spectrin and either vector alone, pCI-p16Ink4a, or pCI-p19Arf and stained with MC540 24 hr later. The samples were analyzed by using a FACScan instrument with gates set on the GFP-positive cells. The data shown are representative of three independent experiments in which these cell lines and two cell lines expressing mutant p53 were analyzed.
In addition, these cells can be transformed by a single, mitogenic oncogene such as Ras without the collaboration of immortalizing oncogenes like Myc and E1A (10, 11, 13, 14, 18, 35). Our observations with v-abl-stimulated lymphocytes suggest that these phenomena are not unique to fibroblasts; analogous events probably are responsible for the high frequency and broad spectrum of tumors observed in Ink4a/Arf and Arf null mice (18, 35).

The Ink4a/Arf locus encodes two proteins, p16\(^{\text{Ink4a}}\) and p19\(^{\text{Arf}}\), both of which have tumor-suppressor functions in appropriate settings (10, 18, 21, 30, 35, 41). However, p19\(^{\text{Arf}}\) appears to be the product involved in crisis induction. The strong correlation between high levels of Ink4a/Arf locus products and the expression of mutant p53 in our transformants mirrors results obtained with fibroblasts lacking Arf (13, 14, 18). Overexpression of p19\(^{\text{Arf}}\) in cells induces apoptosis in a p53-dependent fashion while overexpression of p16\(^{\text{Ink4a}}\) does not significantly alter their health or cell cycle status (A.R. and N.R., unpublished data). This latter result may reflect the high levels of CDK-4 and -6 present in the cells; even when all of the p16 present is bound to these proteins, a large proportion of the CDKs remain complexed to cyclin D3 and the activity of cyclin D3 remains high (unpublished data). Finally, the observation that primary transformants from Tp53\(^{-/-}\) mice fail to undergo crisis (I.U., A.R., and W.R., unpublished data) is consistent with a critical role for the recently described p19\(^{\text{Arf}}\)-p53 regulatory loop (13–15).

A key event in the p19\(^{\text{Arf}}\)-mediated response is up-regulation of p19\(^{\text{Arf}}\) expression (14, 18). In fibroblasts, overexpression of c-Myc, E1A, or probably other immortalizing oncogenes can increase p19\(^{\text{Arf}}\) expression, at least in part through effects on E2F transcription factors (13, 14). The link between v-Abl expression and c-Myc activation is well established (42–45), suggesting a mechanism by which p19\(^{\text{Arf}}\) expression can be activated. However, about half of all primary transformants do not seem to up-regulate p19\(^{\text{Arf}}\), even though activation of c-Myc appears to be an obligatory step in v-Abl-mediated transformation (42, 43, 45). Analyses of 10 transformants that do not express abundant Ink4a/Arf locus products reveal that all of them retain at least one copy of the Ink4a/Arf locus. In addition, these copies usually lack evidence of methylation (A. Halgren, A.R., and N.R., unpublished data). These transformants may have defects in other cellular components required to activate expression of the locus. Interestingly, these transformants still undergo crisis, suggesting that at least one other cellular defense pathway can mediate this response.

At least some primary transformants display high levels of p19\(^{\text{Arf}}\) expression before cells expressing mutant p53 emerge. Because Tp53 mutations initially were detected by using resistance to radiation-induced apoptosis, survival of as few as 1–5% of the treated cells can be monitored readily. This feature and the strong correlation between resistance to radiation-induced apoptosis and mutant p53 in Ab-MLV-transformed pre-B cells (8) make it unlikely that large numbers of cells harboring mutant forms of p53 were present before their detection. These data suggest that one consequence of high p19\(^{\text{Arf}}\) expression is selection of cells carrying Tp53 mutations. Consistent with this idea, only 2 of 40 transformants tested express mutant p53 in the absence of high p19\(^{\text{Arf}}\) levels and only 1 expressed high p19\(^{\text{Arf}}\) and wt p53. Fibroblasts from mice heterozygous for Arf or Tp53 usually lose expression or function of one but not both of these proteins as they emerge from crisis (18). In addition, tumor-associated mutational inactivation of p53 has not been observed in many melanomas and fibrosarcomas derived from Ink4a/Arf null mice (ref. 41; L. Chin and R.A.D., unpublished observations) and occurs rarely in Arf null mice (18). Finally, a revisited analysis of several types of human cancers showed that dual inactivation of ARF and TP53 is exceptionally rare (11).

The effects of p19\(^{\text{Arf}}\) expression coupled with analyses of Tp53 null mice (I.U., A.R., and N.R., unpublished data) and patterns of Tp53 mutation in Ab-MLV-transformed pre-B cells suggest a model in which v-Abl expression, through activation of c-Myc, leads to p19\(^{\text{Arf}}\) up-regulation. p19\(^{\text{Arf}}\) activates p53 (11, 12, 15), inducing the apoptotic crisis observed in primary transformants. In parallel with these events, v-Abl expression also activates Ras, the product of a classical mitogenic oncogene (46, 47), along with other growth-stimulatory pathways (reviewed in refs. 3 and 48); such signals, known to temper p53-mediated apoptosis in other settings (18, 35, 49–52), counter-balance the protective cellular response and allow some cells to survive crisis. However, as noted earlier, the p19\(^{\text{Arf}}\)-p53 regulatory loop does not appear to be the only mechanism by which crisis is induced in primary transformants. Identifying this alternate pathway and determining its role, along with that of the p19\(^{\text{Arf}}\)-p53 regulatory loop in tumor induction, represents a next step toward understanding the multistep process by which tumors arise in this and other systems.

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