

Differential effects of p19^{Arf} and p16^{Ink4a} loss on senescence of murine bone marrow-derived preB cells and macrophages

David H. Randle*[†], Frederique Zindy*, Charles J. Sherr*^{†‡}, and Martine F. Roussel*^{†§}

*Department of Tumor Cell Biology and [†]Howard Hughes Medical Institute, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105; and [‡]Department of Molecular Sciences, University of Tennessee College of Medicine, Memphis, TN 38163

Edited by Owen N. Witte, University of California, Los Angeles, CA, and approved June 19, 2001 (received for review May 2, 2001)

Establishment of cell lines from primary mouse embryo fibroblasts depends on loss of either the *Arf* tumor suppressor or its downstream target, the p53 transcription factor. Mouse p19^{Arf} is encoded by the *Ink4a-Arf* locus, which also specifies a second tumor suppressor protein, the cyclin D-dependent kinase inhibitor p16^{Ink4a}. We surveyed bone marrow-derived cells from wild-type, *Ink4a-Arf*-null, or *Arf*-null mice for their ability to bypass senescence during continuous passage in culture. Unlike preB cells from wild-type mice, those from mice lacking *Arf* alone could be propagated indefinitely when placed onto stromal feeder layers engineered to produce IL-7. The preB cell lines remained diploid and IL-7-dependent and continued to express elevated levels of p16^{Ink4a}. By contrast, *Arf*-null bone marrow-derived macrophages that depend on colony-stimulating factor-1 for proliferation and survival in culture initially grew at a slow rate but gave rise to rapidly and continuously growing, but still growth factor-dependent, variants that ceased to express p16^{Ink4a}. Wild-type bone marrow-derived macrophages initially expressed both p16^{Ink4a} and p19^{Arf} but exhibited an extended life span when p16^{Ink4a} expression was extinguished. In all cases, gene silencing was accompanied by methylation of the *Ink4a* promoter. Therefore, whereas *Arf* loss alone appears to be the major determinant of establishment of murine fibroblast and preB cell lines in culture, p16^{Ink4a} provides an effective barrier to immortalization of bone marrow-derived macrophages.

cell cycle checkpoints | cyclin-dependent kinase inhibitors

Primarily mammalian cells in culture exhibit a limited proliferative capacity, after which they senesce (1, 2). In proliferating human somatic cells in which active telomerase is not normally expressed, telomere erosion from the ends of chromosomes serves as a “mitotic clock” that counts cell divisions. After a finite number of division cycles, ensuing telomere dysfunction activates cell cycle checkpoint controls to trigger replicative arrest (“senescence”). Abrogation of the tumor suppressive functions of the retinoblastoma protein (Rb) and p53 transcription factor bypasses this checkpoint arrest and endows cells with an extended life span. However, progressive telomere shortening ultimately leads to gross chromosomal instability and apoptosis (“crisis”) from which few if any cells survive. To escape crisis, cells must restabilize their telomeres, either by reactivating telomerase or by exploiting alternative recombinational mechanisms for telomere maintenance. The relative inability of normal human cells in culture to bypass both senescence and crisis likely accounts for their rare establishment as continuously proliferating cell lines (reviewed in refs. 3 and 4).

Cultured rodent cells behave differently. Laboratory mice are endowed with telomeres 5- to 10-fold longer than those of human chromosomes (5), and telomerase activity can be detected in many somatic cells (6). Indeed, mice lacking the gene encoding the telomerase RNA subunit are perfectly healthy and reveal consequences of telomere dysfunction only when interbred through multiple sequential generations (7). Yet, despite the

apparent absence of a telomerase-based mitotic clock, explanted mouse embryo fibroblasts (MEFs) undergo senescence much more rapidly than human skin fibroblasts. In MEFs, the induction of inhibitors of cell cycle progression represents the major telomere-independent barrier to cellular immortalization. One hypothesis is that senescence of cultured rodent cells reflects the activation of p53- and Rb-dependent cell cycle checkpoints in response to nonphysiologic conditions of tissue culture per se (“culture shock”) (4, 8). Human cells likely respond in this manner as well, but as a longer-lived species, their cells may have evolved a more effective means of insulating themselves from stresses imposed by the culture environment. This idea has gained support from experiments in which specific alterations in culture conditions greatly extended the life span of both human and rodent cells and in some instances guaranteed seemingly continuous proliferation of explanted primary rodent cells (9–11).

The *Ink4a-Arf* locus encodes two unrelated proteins, p16^{Ink4a} and p19^{Arf}, encoded in part by alternative reading frames within exon 2 of the gene (12). These proteins are tumor suppressors that brake the cell cycle by modulating the activities of Rb and p53, respectively (13). The p16^{Ink4a} protein inhibits the cyclin D-dependent kinases, Cdk-4 and Cdk-6, preventing them from phosphorylating and inactivating Rb (14). The unrelated p19^{Arf} protein is induced in response to an elevated threshold of mitogenic signals that arise from overexpression of c-Myc, E2F-1, E1A, Ras, v-Abl, and other oncoproteins (15–19). p19^{Arf} binds and inactivates functions of the p53 negative regulator, Mdm2, thereby potentiating p53 activity and inducing p53-dependent growth arrest or apoptosis, depending on the biologic context (20–23).

Although not detected during mouse embryonic development, p16^{Ink4a} and p19^{Arf} are induced and accumulate progressively as explanted MEFs are passaged in culture (24). Spontaneously immortalized cell lines derived from primary MEF cultures exhibit either p53 or *Arf* loss of function in a mutually exclusive manner (24). Moreover, MEFs derived from mice lacking *Arf* alone or *Ink4a-Arf* fail to undergo any detectable phase of replicative senescence (25, 26), although those from *Arf*-null mice continue to express high levels of p16^{Ink4a}. Therefore, of the two products encoded by the *Ink4a-Arf* locus, p19^{Arf} plays the dominant role in preventing MEF immortalization.

Different genetic events may well govern the immortalization of other cell types in culture, and many lines of evidence argue

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: MEFs, murine embryonic fibroblasts; MSP, methylation-specific PCR; CSF-1, colony-stimulating factor 1; BM, bone marrow.

[§]To whom reprint requests should be addressed at: Department of Tumor Cell Biology, DTRT 5006C, 332 North Lauderdale, Memphis, TN 38105. E-mail: martine.roussel@stjude.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

that p16^{Ink4a} plays a major role in imposing a proliferative barrier, particularly in various human cell strains (3, 4, 27). Here, we show that, whereas elimination of *Arf* is apparently sufficient to establish bone marrow (BM)-derived preB cells as continuously proliferating cell lines in culture, BM-derived macrophages preferentially silence *Ink4a* without losing expression of the closely linked Cdk inhibitor, p15^{Ink4b}, or of p19^{Arf}.

Materials and Methods

Cells and Culture Conditions. Cells harvested from the femurs and tibias of mice were explanted into cultures onto NIH 3T3 feeder layers in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with 5% FCS/55 μ M 2-mercaptoethanol/2 mM glutamine, penicillin, and streptomycin (GIBCO) (28, 29) and phenotyped as described (30). The NIH 3T3 subclone was engineered to secrete human recombinant interleukin-7 (DNAX) and provides efficient stromal cell support for culturing either primary preB cells or IL-7-dependent cell lines (ref. 30 and data not shown). Two-thirds of the preB cells were removed at 3-day intervals, medium was replenished, and residual cells were allowed to proliferate until growth ceased (1–2 months for cultures of wild-type cells) or until experiments with *Arf*-null cells were terminated. At day 10 after explantation, some preB cells were transferred from feeders to IL-7-conditioned liquid medium without stromal support (30). Cells were plated in triplicate at a density of 2.5×10^5 /ml in 6-well plates, counted every 2 days, and rediluted to the starting density in fresh medium every fourth day. Under these conditions, IL-7 was not rate-limiting for cell proliferation.

Primary cultures of BM-derived macrophages were generated from 4- to 8-week-old *Arf*-null, *Ink4a-Arf*-null, and wild-type mice. Cells were seeded at 1×10^6 cells per ml in DMEM (BioWhittaker) supplemented with 15% FCS/5.5 μ M 2-mercaptoethanol/2 mM glutamine, penicillin, and streptomycin, and L cell-derived colony-stimulating factor-1 (CSF-1) (31). Nonadherent cells were removed 24 h later, resuspended at the same cell density in fresh medium, replated, and then removed after a second 48-h incubation to separate progenitor cells from adherent phagocytes and fibroblasts. Nonadherent cells were seeded a final time at the same density and propagated for 3–4 days until confluent [Passage (P)-1]. Staining with antibodies to Mac-1 (M1/70 PharMingen) and F4/80 (32) revealed that all cells expressed the macrophage markers. Scraped cells were replated at 4×10^5 per ml, fed with L cell-conditioned-medium every 2 days, counted when confluent, diluted 1:5 in fresh medium before replating, and maintained on this protocol throughout the experiment. Growth kinetics were determined for six independent cultures of each genotype counted in triplicate. The mouse CSF-1-dependent macrophage cell line BAC1.2F5 was maintained as described (32); preB cells transformed by p210^{BCR-Abl} were a gift from Owen Witte (University of California, Los Angeles). MEF (clone 10) containing mutant p53 and expressing high levels of both p19^{Arf} and p16^{Ink4a} was derived previously (24).

Irradiation and Apoptosis Assays. PreB cells were suspended at 10^6 cells per ml in medium containing IL-7 and were irradiated with 4 Gy from a cesium source. Cell viability was determined 24 h later by analysis of cells stained with annexin V and propidium iodide (33). BM-derived macrophages were irradiated with 5 Gy, harvested, lysed, and analyzed by immunoblotting for induction of p53 and Mdm2.

Immunoblotting. Frozen cell pellets were lysed in ice-cold EBC buffer (34). Nuclei and debris were sedimented, and protein quantified by using a BCA Protein Assay Kit (Pierce). Proteins (200 μ g per lane) separated on 12.5% denaturing polyacrylamide gels containing SDS were transferred to nitrocellulose (Osmon-

ics, Westborough, MA) and visualized (Western Blot Chemiluminescence Reagent, NEN Life Science, Boston) after blotting with affinity-purified rabbit polyclonal antisera specific to the C termini of p19^{Arf} (12) or p16^{Ink4a} (35), with antibody 2A10 to Mdm2 (15), or with commercial antibodies to p53 (Ab7, Calbiochem) or to α -tubulin (T-5168 Sigma). Rabbit polyclonal antiserum (RAE) to the p15^{Ink4b} C terminus was used to detect the protein by sequential precipitation and blotting (24).

RNA Analysis. Total RNA was extracted from cultured macrophages by using RNazol B (Tel-Test, Friendswood, TX). RNA (50 μ g/lane) was separated electrophoretically in formaldehyde-containing gels, blotted to Hybond-N+ nylon hybridization membranes (Amersham Pharmacia Biotech) and detected by using a 110-bp [³²P]-labeled probe specific for exon 1 α of the *Ink4a-Arf* locus (35).

Methylation-Specific PCR (MSP). Bisulfite modification of genomic DNA and MSP were performed (36) with primer sets designed to amplify PCR products from the *Ink4a* promoter region and exon 1 α . Primers that detected methylated DNA yielded a 199-bp PCR product (nucleotide positions 8–207 according to GenBank accession no. L76150), whereas those that hybridized to unmethylated templates amplified a 197-bp product (nucleotide positions 10–207). Retention of exon 1 α was confirmed by amplifying untreated genomic DNA with wild-type primers to produce a 201-bp fragment (nucleotide positions 6–207). Primer sequences were: (methylated) 5'-ATACGATTGGGCGATTGGGCG-3' (sense) and 5'-CACCTAAATCGAAATACGACCGA-3' (antisense) (unmethylated); 5'-ATGATTGGGTGATTGGGTG-3' (sense) and 5'-CATCACCTAAATCAAATA-CAA-3' (antisense); and (wild-type) 5'-TCACACGACTGGGCGATTGG-3' (sense) and 5'-CACCTGAATCGGGTACGAC-3' (antisense). Reactions were initiated at 95°C for 7.5 min, *Taq* polymerase (Promega) was added, and DNA was amplified for 30 cycles of denaturation (95°C, 30 s), annealing (61°C, methylated; 51°C, unmethylated, and 56°C, wild-type, 30 s) and extension (72°C, 45 s) by using a thermal cycler (5331 Mastercycler, Brinkmann). PCR products were visualized on 2.0% agarose gels in Tris-acetate/EDTA electrophoresis buffer containing ethidium bromide (0.5 ng/ml).

Results

p19^{Arf} Deficiency Prevents Senescence of Primary preB Cells. We derived long-term IL-7-dependent B lymphocyte cultures from bone marrow of age-matched wild-type and *Arf*-null mice. When cultured on NIH 3T3 feeder cells engineered to express IL-7, myeloid progenitors cannot survive, and a homogeneous population of preB cells (>90%) grows out after 10 days. PreB cells from wild-type mice could be continuously expanded on feeder layers, initially doubling every 2–3 days, but they underwent replicative senescence within 1–2 months (28). In contrast, *Arf*-null preB cells proliferated continuously and at significantly faster rates doubling every 24 h, even after 3 months in culture when many such experiments were terminated (data not shown). When wild-type preB cells were removed from feeder layers after 10 days of culture and propagated under more stringent liquid culture conditions in the presence of saturating quantities of IL-7 but without other stromal factors, the cells proliferated more slowly, doubling every 5 days. After 2 weeks in liquid medium, their proliferation ceased and cells gradually died (Fig. 1A). In contrast, *Arf*-null preB cells doubled every 1.5 days and proliferated continuously throughout the duration of the experiment (Fig. 1A). Indeed, the generation time of *Arf*-null preB cells was indistinguishable from that of an established, transformed and tumorigenic preB cell line expressing the p210^{BCR-Abl} oncoprotein.

Arf-null MEFs become established immediately after explantation

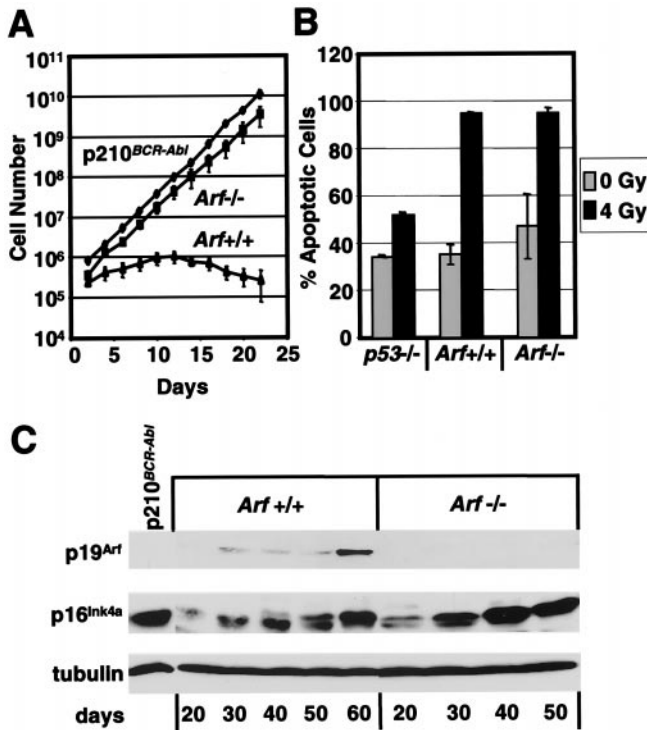


Fig. 1. p19^{Arf} deficiency bypasses senescence of preB cells. (A) Kinetics of cell growth were determined for five independently derived lines of *Arf*-null (■) and wild-type (▲) preB cells in IL-7-containing medium and compared with a previously immortalized preB cell line transformed by *BCR-Abl* (◆). (B) After exposure to 4 Gy γ -irradiation, preB cells of the indicated genotypes (Bottom) were cultured for 24 h and stained with annexin V and propidium iodide to determine cell viability. (C) PreB cells maintained on IL-7-expressing feeder layers were harvested and lysed at 10-day intervals (days, Bottom) over a 2-month period. Results are compared with those obtained with cells transformed by *BCR-Abl* (p210). Proteins were detected by direct immunoblotting by using antibodies raised against p19^{Arf} and p16^{Ink4a}, as indicated in the left margin. Detection of α -tubulin was used to confirm equal protein loading.

tation into culture, even though they accumulate high levels of p16^{Ink4a} on further passage (26). Similarly, both wild-type and *Arf*-null preB cells accumulated p16^{Ink4a} protein as they were passaged (Fig. 1C), even when maintained on IL-7-producing feeder layers. Late passage *Arf*-null preB cells continued to proliferate at the same high rate, despite robust p16^{Ink4a} expression. Increased p19^{Arf} protein expression was observed as wild-type preB cells senesced (Fig. 1C). Cells immortalized by *BCR-Abl* also expressed high levels of p16^{Ink4a}, although selectively eliminating expression of p19^{Arf} (Fig. 1C), consistent with previous observations that *v-Abl* expression in preB cells selects for loss of either p19^{Arf} or p53 function (19). Therefore, of the two proteins encoded by the *Ink4a-Arf* locus, only p19^{Arf} is required to impose replicative arrest in preB cells.

Five separate *Arf*-null preB cell lines maintained on IL-7-producing feeders for at least 3 months remained dependent on IL-7 for growth and survival. G₁ phase cells remained diploid, which is characteristic of immortalized *Arf*-null MEFs and distinguishes them from established fibroblast cell lines that lack p53 function and rapidly become tetraploid (24, 26). PreB cell lines expressed very low levels of wild-type p53, as determined with antibodies that distinguish normal from mutant isoforms (data not shown). Because mutant p53 is unable to transcriptionally activate its negative regulator Mdm2, mutant forms of p53 are stable. The failure to accumulate p53 protein also implied that p53 mutations had not occurred. When preB cells were exposed to a low dose of γ -irradiation (4 Gy), and apoptotic

cells were stained 24 h later with annexin V, *Arf*-null cells, like their wild-type counterparts, were highly sensitive and rapidly underwent apoptosis (Fig. 1B), whereas p53-null preB cells were markedly resistant (Fig. 1B) (37). Collectively, these data imply that *Arf*-null preB cells can continue to proliferate at a high rate in culture without sustaining p53 or p16^{Ink4a} loss of function.

p16^{Ink4a} Down-Regulation Accompanies Establishment of Bone Marrow-Derived Macrophage Cell Lines. Murine macrophage cell lines have previously been derived after infection of peritoneal macrophages or myeloid progenitors with replication-defective simian virus (SV)40 and propagation of infected cells in CSF-1-containing medium. Established macrophage cell lines emerged 1–2 months after viral infection but were aneuploid and no longer depended on CSF-1 for proliferation or survival (38, 39). To date, only one CSF-1-dependent, diploid murine macrophage cell line has been derived after infection of myeloid progenitors with SV40 virus (32, 40). These BAC1.2F5 cells sustained bi-allelic deletion of the *Ink4a-Arf* locus, but lack detectable SV40 viral DNA (35).

To determine whether primary *Arf*-null macrophages, like *Arf*-null murine preB cells and MEFs, would exhibit an extended life span in culture, we explanted BM-derived macrophages into medium containing CSF-1. Under these conditions, established *Ink4a-Arf*-null BAC1.2F5 macrophages grew with a constant doubling time (T_d) of 1.3 days throughout serial passage (Fig. 2A and Table 1). We emphasize that these cells have been propagated in culture for many years and have been periodically subcloned to retain CSF-1 dependence; they therefore have had ample opportunity to acquire additional growth-promoting mutations during their long passage history. Strikingly, *Ink4a-Arf*-null macrophages explanted directly from mice also maintained a constant doubling time throughout the duration of the experiment ($T_d = 1.9$ days) that approached that of BAC1.2F5 cells. Wild-type macrophages proliferated much more slowly ($T_d = 7.1$ days) during their first month in culture (Fig. 2A and Table 1). Macrophages lacking *Arf* alone proliferated faster than wild-type cells during this period ($T_d = 3.3$ days) but grew slower than their *Ink4a-Arf*-null counterparts (Table 1). Eventually, however, more rapidly proliferating cells emerged from both the *Arf*-null and wild-type populations, and these exhibited faster doubling times for the remainder of the experiment. The latter cultures were maintained for an additional 3 months without any further change in proliferative rate. Late passage cells of all genotypes remained growth factor-dependent, retained macrophage cell surface markers, and demonstrated phagocytosis of antibody-coated avian erythrocytes (data not shown). Together, these data suggested (i) that both p16^{Ink4a} and p19^{Arf} cooperate in preventing establishment of murine macrophage cell lines, and (ii) that wild-type and *Arf*-null macrophages likely sustained additional mutations that facilitated their adaptation to culture.

Given the initial differences in generation times of explanted *Ink4a-Arf*-null and *Arf*-null macrophages, an obvious possibility was that additional mutations accelerating the proliferation of *Arf*-null cells might reflect subsequent loss of expression of p16^{Ink4a}. Immunoblotting analyses of cell lysates revealed that the emergence of faster growing variants from cultures of either wild-type or *Arf*-null macrophages correlated with down-regulation of p16^{Ink4a} protein expression (Fig. 2B). Loss of p16^{Ink4a} from wild-type cells between 36 and 50 days of culture (Fig. 2B) was accompanied by an abrupt shortening of cell generation time during this interval (Fig. 2A; Table 1), whereas similar changes in *Arf*-null cells occurred more rapidly between days 28–35 (Fig. 2A and B; Table 1). The p19^{Arf} protein continued to accumulate in wild-type macrophages even after faster growing cells emerged (Fig. 2C), and the latter variants proliferated more slowly than cells lacking both p19^{Arf} and p16^{Ink4a} (Table 1). Expression of the 15 kDa product of the

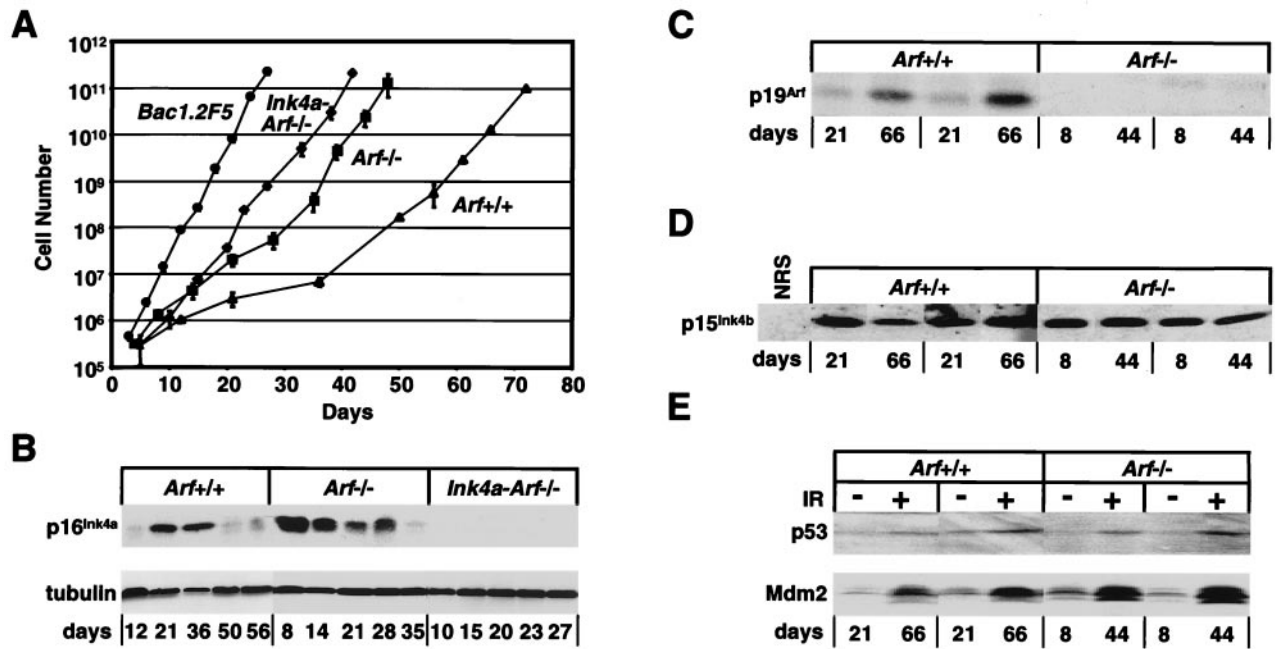


Fig. 2. Proliferation kinetics and protein expression in macrophages of different genotypes. (A) BM-derived macrophages cultured in 60-mm diameter dishes were counted when confluent and rediluted 1:5 for the next passage. Data were plotted for six independent cultures derived from *Arf*-null (■), *Ink4a-Arf*-null (◆) and wild-type (▲) mice, and compared with those obtained with the established macrophage cell line, BAC1.2F5 (●). (B) Cells taken at indicated times were assayed for expression of p16^{Ink4a} and α -tubulin by immunoblotting. Macrophages grown for the designated times (days) were lysed and assayed by direct immunoblotting for p19^{Arf} (C) or by sequential immunoprecipitation and blotting for p15^{Ink4b} (D). (E) Macrophages of the indicated genotypes (Top) after different times in continuous culture (Bottom) were exposed to 5 Gy γ -irradiation (+) or left untreated (–) and analyzed by immunoblotting for p53 and Mdm2 protein induction. Representative results with several independently derived populations are shown in C–E.

closely linked *Ink4b* gene was similarly maintained in late passage wild-type and *Arf*-null cells, as determined by sequential immunoprecipitation and blotting (Fig. 2D). All cell populations accumulated p53 and its transcriptional target Mdm2 after γ -irradiation, arguing for retention of functional wild-type p53 (Fig. 2E). Therefore, p16^{Ink4a} expression was eliminated during propagation of both *Arf*-null and wild-type BM-derived macrophages, whereas the *Ink4b* coding region was retained and expressed.

Methylation-Associated Suppression of *Ink4a* Expression in BM-Derived Macrophages. Although initially observed, p16^{Ink4a} protein could no longer be detected in later passage wild-type or *Arf*-null macrophages (Fig. 3A). Similarly, Northern blot analysis performed with a 110-bp exon 1 α cDNA probe that specifically detects *Ink4a* but not *Arf* transcripts (12, 35) revealed no *Ink4a* mRNA at later times (Fig. 3B). We therefore carried out

methylation-specific PCR analysis of bisulfite-modified genomic DNA (36). Although each of the three *Ink4a* coding exons contains a CpG island, only methylation of exon 1 α and the 5' promoter region occurs in cell lines and tumors derived from both humans and mice (41, 42). We designed oligonucleotide primers to amplify a \approx 200-bp region including 75 bp from the 5'

Table 1. Generation times of primary bone marrow-derived macrophages

Genotype	Passage	Days in culture	Doubling time, days
Wild type	P2–P5	1–36	7.1
	P5–P10	36–72	2.6
<i>Arf</i> -null	P2–P6	1–28	3.3
	P6–P10	28–48	1.7
<i>Ink4a-Arf</i> -null	P2–P10	1–42	1.9
BAC1.2F5	P2–P10	1–27	1.3

Macrophages were passaged as described in Fig. 2, and their doubling times were determined at the designated intervals. Because cells were passaged just after they became confluent, passage numbers for cells of different genotypes correspond to different numbers of days in culture. Immortal CSF-1-dependent BAC1.2F5 cells have sustained bi-allelic deletion of the *Ink4a-Arf* locus.

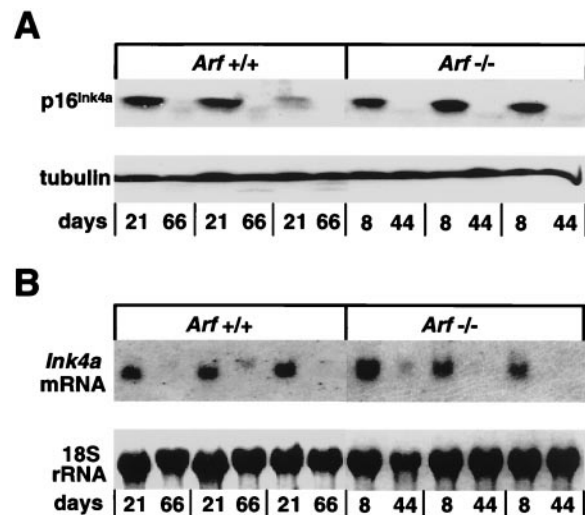


Fig. 3. Transcriptional repression of *Ink4a* in established BM-derived macrophages. (A) Lysates from macrophages after different times in continuous culture were immunoblotted for p16^{Ink4a} and α -tubulin; three independent clones of wild-type and *Arf*-null cells were studied. (B) Northern blot analysis was carried out with RNA extracted from parallel cultures of the same cells described in A. RNA was hybridized with an exon 1 α probe specific for *Ink4a*; 18S rRNA levels were determined by methylene blue staining.

Transcriptional repression of *Ink4a* associated with hypermethylation of the proximal promoter and first coding exon has been previously reported to occur as a telomere-independent mechanism to overcome early senescence in human mammary epithelial cells and keratinocytes (44, 45). Although we believe that such telomere-independent barriers to proliferation occur as a checkpoint response to stress imposed by artificial culture conditions (3, 4), hypermethylation of p16^{Ink4a} occurs in many forms of human cancer (46, 47). We might surmise that in mice, as well as in humans, the loss of *Ink4a* alone (with retention of *Arf*) can contribute to tumor formation in those cell types that rely strongly on p16^{Ink4a} function for checkpoint control. A deeper understand-

ing of this lineage specificity may help explain why *Ink4a* and *Arf* are differentially targeted in human cancers.

We thank Dr. Richard Ashmun for performing flow cytometric analyses, Dr. Tal Teitz for assistance in setting up the MSP analysis, Dr. William Walker for characterization of BM-derived macrophages, Dr. Ronald A. DePinho for generously providing *Ink4a-Arf*-null mice, and Dr. Owen Witte for transformed preB cells. We also gratefully acknowledge the excellent technical assistance of Rose Matthew, Camulous Hornsby, and Esther van de Kamp. This work was supported by National Institutes of Health Grants CA56819 and CA71907 (to M.F.R.), Cancer Core Grant CA21765, and the American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital. C.J.S. is an Investigator of the Howard Hughes Medical Institute.

1. Hayflick, L. & Moorhead, P. S. (1961) *Exp. Cell. Res.* **25**, 585–621.
2. Todaro, G. J. & Green, H. (1963) *J. Cell. Biol.* **17**, 299–313.
3. Wright, W. E. & Shay, J. W. (2000) *Nat. Med.* **6**, 849–851.
4. Sherr, C. J. & DePinho, R. A. (2000) *Cell* **102**, 407–410.
5. Kipling, D. & Cooke, H. J. (1990) *Nature (London)* **347**, 400–402.
6. Prowse, K. R. & Greider, C. W. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 4818–4822.
7. Blasco, M. A., Lee, H.-W., Hande, M. P., Samper, E., Lansdorp, P. M., DePinho, R. A. & Greider, C. W. (1997) *Cell* **91**, 25–34.
8. Wright, W. E. & Shay, J. W. (2001) *Science* **291**, 839–840.
9. Ramirez, R. D., Morales, C. P., Herbert, B.-S., Rohde, J. M., Passons, C., Shay, J. W. & Wright, W. E. (2001) *Genes Dev.* **15**, 398–403.
10. Tang, D. G., Tokumoto, Y. M., Apperly, J. A., Lloyd, A. C. & Raff, M. C. (2001) *Science* **291**, 868–871.
11. Mathon, N. F., Malcolm, D. S., Harrisingham, M. C., Cheng, L. & Lloyd, A. C. (2001) *Science* **291**, 872–875.
12. Quelle, D. E., Zindy, F., Ashmun, R. A. & Sherr, C. J. (1995) *Cell* **83**, 993–1000.
13. Sherr, C. J. (1998) *Genes Dev.* **12**, 2984–2991.
14. Serrano, M., Hannon, G. J. & Beach, D. (1993) *Nature (London)* **366**, 704–707.
15. Zindy, F., Eischen, C. M., Randle, D., Kamijo, T., Cleveland, J. L., Sherr, C. J. & Roussel, M. F. (1998) *Genes Dev.* **12**, 2424–2433.
16. De Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S.-Y., Ferbeyre, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J. & Lowe, S. W. (1998) *Genes Dev.* **12**, 2434–2442.
17. Bates, S., Phillips, A. C., Clarke, P., Stott, F., Peters, G., Ludwig, R. L. & Vousden, K. H. (1998) *Nature (London)* **395**, 124–125.
18. Palmero, I., Pantoja, C. & Serrano, M. (1998) *Nature (London)* **395**, 125–126.
19. Radfar, A., Unnikrishnan, I., Lee, H.-W., DePinho, R. A. & Rosenberg, N. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 13194–13199.
20. Pomerantz, J., Schreiber-Agus, N., Liégeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.-W., Cordon-Cardo, C. & DePinho, R. (1998) *Cell* **92**, 713–723.
21. Zhang, Y., Xiong, Y. & Yarbrough, W. G. (1998) *Cell* **92**, 725–734.
22. Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F. & Sherr, C. J. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 8292–8297.
23. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H. & Peters, G. (1998) *EMBO J.* **17**, 5001–5014.
24. Zindy, F., Quelle, D. E., Roussel, M. F. & Sherr, C. J. (1997) *Oncogene* **15**, 203–211.
25. Serrano, M., Lee, H.-W., Chin, L., Cordon-Cardo, C., Beach, D. & DePinho, R. A. (1996) *Cell* **85**, 27–37.
26. Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G. & Sherr, C. J. (1997) *Cell* **91**, 649–659.
27. Ruas, M. & Peters, G. (1998) *BBA Rev. Cancer* **1378**, F115–F177.
28. Whitlock, C. A. & Witte, O. N. (1987) *Methods Enzymol.* **150**, 275–286.
29. Borzillo, G. V. & Sherr, C. J. (1989) *Mol. Cell. Biol.* **9**, 3973–3981.
30. Eischen, C. M., Weber, J. D., Roussel, M. F., Sherr, C. J. & Cleveland, J. L. (1999) *Genes Dev.* **13**, 2658–2669.
31. Stanley, E. R. (1986) *Methods Enzymol.* **116**, 564–587.
32. Morgan, C., Pollard, J. W. & Stanley, E. R. (1987) *J. Cell. Physiol.* **130**, 420–427.
33. Vermes, I., Haanen, C., Steffens-Nakken, H. & Reutelingsperger, C. (1995) *J. Immunol. Methods* **184**, 39–51.
34. Inoue, K., Wren, R., Rehg, J. E., Adachi, M., Cleveland, J. L., Roussel, M. F. & Sherr, C. J. (2000) *Genes Dev.* **14**, 1797–1809.
35. Quelle, D. E., Ashmun, R. A., Hannon, G. J., Rehberger, P. A., Trono, D., Richter, H., Walker, C., Beach, D., Sherr, C. J. & Serrano, M. (1995) *Oncogene* **11**, 635–645.
36. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D. & Baylin, S. B. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 9821–9826.
37. Strasser, A., Harris, A. W., Jacks, T. & Cory, S. (1994) *Cell* **79**, 329–339.
38. Stone, L. B. & Takemoto, K. K. (1970) *J. Virol.* **6**, 621–627.
39. Mauel, J. & Defendi, V. (1971) *J. Exp. Med.* **134**, 335–350.
40. Schwarzbaum, S., Halpern, R. & Diamond, B. (1984) *J. Immunol.* **132**, 1158–1162.
41. Jones, P. A. & Laird, P. W. (1999) *Nat. Genet.* **21**, 163–167.
42. Malumbres, M., de Castro, I. P., Santos, J., Melendez, B., Mangués, R., Serrano, M., Pellicer, A. & Fernandez-Piqueras, J. (1997) *Oncogene* **14**, 1361–1370.
43. Holland, E. C., Hively, W. P., Gallo, V. & Varmus, H. E. (1998) *Genes Dev.* **12**, 3644–3649.
44. Kiyono, T., Foster, S. A., Koop, J. I., McDougall, J. K., Galloway, D. A. & Klingelutz, A. J. (1998) *Nature (London)* **396**, 84–88.
45. Brenner, A. J., Stampfer, M. R. & Aldaz, C. M. (1998) *Oncogene* **17**, 199–205.
46. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M. & Issa, J.-P. (1998) *Adv. Cancer Res.* **72**, 141–196.
47. Esteller, M., Corn, P. G., Baylin, S. B. & Herman, J. G. (2001) *Cancer Res.* **61**, 3225–3229.