Heterogeneous activation of p19Arf in pulmonary artery smooth muscle cells

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Heterogeneous activation of p19Arf in pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 300: L642–L647, 2011. First published January 7, 2011; doi:10.1152/ajplung.00117.2010.—p19Arf is a tumor suppressor that leads to cell cycle arrest or apoptosis by stabilizing p53. p19Arf is not critical for cell cycle regulation under normal conditions, but loss of p19Arf is seen in many human cancers, and a murine p19Arf knockout model leads to malignant proliferation and tumor formation; its role in controlling nonmalignant proliferation is less defined. To examine this question, pulmonary artery smooth muscle cells (PASMC) were expanded in culture from a transgenic mouse in which the coding sequence of the p19Arf gene was replaced with a CDNA encoding green fluorescent protein (GFP), leaving the promoter intact. During the first 10 days in culture, wild-type, heterozygous, and knockout PASMC grew similarly, but, by day 14, p19Arf-deficient PASMC proliferated faster than p19Arf heterozygous or wild-type cells; reexpression of p19Arf prevented the increased proliferation. This time course correlated with activation of the p19Arf promoter, as indicated by the appearance of GFP positivity in p19Arf-deficient PASMC. By day 42, ~80% of p19Arf-deficient cells were GFP-positive. When GFP-positive, p19Arf-deficient cells were sorted and subcultured separately, they remained GFP-positive, indicating that once cells had activated the p19Arf promoter, the promoter remained active in those and all subsequent daughter cells. In contrast, GFP-negative p19Arf-deficient cells gave rise to a combination of GFP-positive and -negative daughter cells over time. These results suggest that a subpopulation of PASMC are resistant to the signals that activate the p19Arf promoter, an event that would normally target these cells for arrest or cell death.

Materials. Monoclonal mouse anti-smooth muscle α-actin antibody (catalog no. A-2547) and monoclonal mouse anti-β-actin antibody (catalog no. A-5441) were purchased from Sigma (St. Louis, MO); mouse monoclonal anti-GFP antibody [B-2, catalog no. sc-9996, horseradish peroxidase (HRP)-conjugated] from Santa Cruz Biotechnology (Santa Cruz, CA); anti-mouse IgG HRP-linked whole antibody (catalog no. NA931V, from sheep) from GE Healthcare (Little Chalfont, Buckinghamshire, UK); DMSO, trypsin-EDTA, and L-glutamine from GIBCO (Grand Island, NY); FBS from Atlanta Biologicals (Lawrenceville, GA); HyBond-P membrane from Amer sham (Buckinghamshire, UK); SuperSignal West Dura from Pierce Chemical Co (Rockford, IL); FuGENE 6 transfection reagent (catalog no. 11 814 443 001) from Roche Diagnostics (Indianapolis, IN); calcein-AM from Molecular Probes (catalog no. C3099); and Annexin V-Phycoerythrin (PE) Apoptosis Detection Kit I from BD Pharmingen (catalog no. 559763).

Cells. Drs. Zindy and Sherr (St. Jude’s Hospital, Memphis, TN) graciously provided the p19Arf transgenic mice. To generate the mice, they replaced the coding sequences of exon 1B of the mouse cellular Arf gene with a cDNA encoding GFP (29). This produced an Arf-null mouse on a C57BL/6 background in which GFP expression was driven by the intact Arf promoter. Heterozygotes were mated, yielding litters of p19Arf wild-type, heterozygous, and knockout pups. Smooth muscle cells were isolated by elastase and collagenase digestion of main (extralobar) pulmonary arteries from adult (5- to 8-wk-old) mice, as previously described (1, 2). Cells were used between pas-
sages 1 and 9. Greater than 95% of cells stained positive for smooth muscle α-actin (mouse monoclonal antibody, Sigma) at each passage and were negative for vascular endothelial, pan, and nonepithelial cadherin. The breeding and experimental protocols were approved by the University of South Alabama Institutional Animal Care and Use Committee.

PASMC from three genotypes (p19^Arf^+/+ , p19^Arf^-/-, and p19^Arf^-/-) were cultured separately in DMEM-F-12 medium, 10% FBS, and 2 mmol of l-glutamine for up to 49 days. Cells were harvested by 0.05% trypsin-0.53 mM EDTA digestion and counted in triplicate with a particle counter (model Z1, Coulter Electronics). For cell visualization, calcein-AM (5 μmol/l) was added directly to the cell cultures for 10 min. The intracellular esterase activity in live cells converts the nonfluorescent cell-permeable calcein-AM to the intensely fluorescent, cell-impermeable calcein, with excitation at 495 nm and emission at 515 nm. This produces a uniform green fluorescence in live cells, which makes them visible under a fluorescent microscope.

Flow cytometry analysis. Flow cytometry was used to sort cells on the basis of reporter gene [GFP or DsRed (DsRed)] expression for subculture experiments to quantify the number of cells expressing reporter genes and to determine the percentage of annexin V-positive cells. Cells were harvested by 0.05% trypsin-0.53 mM EDTA digestion, washed, resuspended in culture medium, and analyzed directly by FACScan for fluorescent protein(s) expression in the University of South Alabama Flow Cytometry Core. Excitation and emission were 558 and 583 nm, respectively, for DsRed, 488 and 578 nm, respectively, for annexin V-PE (Molecular Probes), and 488 and 507 nm, respectively, for GFP.

For p19^Arf^ overexpression, we cloned the p19^Arf^ mouse cDNA (gift of F. Zindy) into a bicistronic vector, together with DsRed as a reporter. A vector containing only DsRed was used as a control. Cells were transiently transfected using FuGENE 6 transfection reagent according to the manufacturer’s instructions. Cells expressing DsRed were identified by flow cytometry.

Real-time PCR. Total cellular RNA was isolated using the RNeasy Mini Kit (catalog no. 74104, Qiagen). The levels of p19^Arf^ or Bmi-1 mRNA in preparations were determined by quantitative PCR using the iScript One-Step RT-PCR kit [with SYBR Green (catalog no. 170-8893, Bio-Rad)] following the manufacturer’s instructions. The quantitative PCR data were normalized to the level of 28S RNA. Amino acid sequences were as follows: catgtgttgaggctagagagg (forward) and gcagcctgtagtgacagaggg (reverse) for p19^Arf^ (mouse); ctcgtagtggattgtaagagc (forward) and gagggtgagatgtcttttgtc (reverse) for BMI-1 (mouse), set 1; and aatgtgtgtcctgtgtggaggg (forward) and tgcgctgtggcagttgag (reverse) for Bmi-1 (mouse), set 2.

Western blotting. Equal amounts of protein obtained from whole cell lysates were separated by SDS-PAGE under reducing conditions (5 mmol/l DTT). The proteins were then transferred to a polyvinylidene difluoride membrane, probed with appropriate antibodies (see Materials), detected with anti-mouse secondary antibodies conjugated to HRP, and visualized using the SuperSignal chemiluminescence detection system (Pierce) according to the manufacturer’s instructions.

Statistical analysis. Values are means ± SE. Cell growth was compared using ANOVA combined with Fisher’s post hoc analysis; P < 0.05 was considered significant. Annexin V-PE analysis, real-time PCR data, and protein expression levels were compared using a two-tailed unpaired t-test; P < 0.05 was considered significant.

RESULTS

p19^Arf^-deficient PASMC proliferate more than wild-type PASMC. The left and right main pulmonary arteries were dissected free from p19^Arf^- wild-type, heterozygous, and knockout C57BL/6 littersmates, digested, and plated in DMEM-F-12 medium with 10% FBS. After the cells were allowed to adhere for 48 h, they were trypsinized and replated at 1 × 10^5 cells/well and grown for 7 days. Cells were then trypsinized, counted, and replated at 1 × 10^5 cells/well every 7 days for 49 days.

Figure 1 demonstrates the weekly changes in PASMC growth through day 49. Between days 0 and 14, the different genotypes had similar growth curves. By day 14, the growth curves began to diverge, with p19^Arf^- knockout cells proliferating faster than wild-type PASMC and heterozygotes. By day 49, there was a marked increase in the number of PASMC derived from an initial pulmonary artery harvested from the p19^Arf^- knockout animal compared with the number obtained from a wild-type pulmonary artery (Fig. 1A). The loss of a single p19^Arf^- allele also provided a proliferative advantage over wild-type PASMC. Figure 1B confirms that heterozygotes have half the p19^Arf^- mRNA of wild-type PASMC and that no p19^Arf^- mRNA was detected in cells grown from knockout mice. In parallel experiments, cells were plated in a six-well plate, grown in 10% FBS, and then stained with calcein-AM, a membrane-permeable dye that allows visualization of live cells. Figure 1C demonstrates that while size and number of colonies were similar for wild-type and knockout cells in the first 7 days, these similarities disappeared over time. By day 42, p19^Arf^- knockout cells no longer formed colonies but, rather, rapidly proliferated as a single sheet. The growth of wild-type cells was markedly reduced in comparison.

p19^Arf^- promoter in PASMC in culture is activated between days 7 and 14. Growth curves for the different phenotypes began to diverge by day 14. We examined whether this correlated with activation of the p19^Arf^- promoter. In this animal, the gene for p19^Arf^- has been replaced with GFP. Therefore, when the p19^Arf^- promoter is activated, p19^Arf^-deficient homozygous and heterozygous cells become GFP-positive. This can be detected with flow cytometry and Western blotting. Figure 2A demonstrates that, at day 7, only a few of the p19^Arf^-deficient PASMC were GFP-positive. The number of GFP-positive cells began to increase between days 7 and 14 and continued to increase through day 42, at which time they constituted ~80% of cells, indicating that the p19^Arf^- promoter has been activated in the majority of cells. Western blotting for GFP confirmed these observations (Fig. 2B).

Figure 2A indicates that while GFP-positive PASMC accumulated in the p19^Arf^-deficient culture, the percentage of GFP-positive heterozygous (p19^Arf^-/-) cells remained ~3–4% through day 42. In p19^Arf^-/- PASMC, activation of the p19^Arf^- promoter should produce GFP from one allele, whereas the other allele will generate p19^Arf^-. We questioned whether the single wild-type p19^Arf^- allele was sufficient to induce apoptosis or growth arrest in these cells and, thus, prevent the accumulation of GFP-positive cells. We stained p19^Arf^- wild-type, heterozygous, and deficient PASMC for annexin V, a membrane protein that is expressed in apoptotic and necrotic cells. Using flow cytometry, we then analyzed cells for GFP and annexin V. Figure 3 demonstrates that almost all GFP-positive p19^Arf^-deficient cells were annexin V-negative, indicating that, despite activation of the p19^Arf^- promoter, they were not undergoing apoptosis. In contrast, >50% of the GFP-positive heterozygous cells were annexin V-positive, indicating that they were undergoing apoptosis. This suggests that the failure of GFP-positive heterozygous cells to accumulate was due to...
the activation of the wild-type allele, which expressed sufficient p19Arf to promote apoptosis in these PASMC. Demonstration of a 10-fold-higher p19Arf mRNA level in heterozygous cells that were GFP-positive than in heterozygous cells that were GFP-negative supports this observation (Fig. 3B).

To determine whether replacing p19Arf in the p19Arf knockout PASMC slowed their growth or induced apoptosis, we transiently transfected p19Arf-deficient cells with a DsRed-expressing plasmid encoding the mouse p19Arf gene. The percentage of DsRed-positive cells was then tracked over 5 days using flow cytometry. Figure 4 demonstrates that, by day 3, ~35% of p19Arf-deficient PASMC transfected with the empty (control) vector expressed DsRed; this level remained relatively constant over the next 2 days. In contrast, only 7% of cells transfected with the plasmid containing both DsRed and p19Arf were positive at day 2; this level decreased to <5% by day 5. These results indicate that restoring p19Arf to these p19Arf-deficient cells induced apoptosis.

**Activation of p19Arf promoter persists in p19Arf-deficient cells.** p19Arf does not control the cell cycle during normal growth but is upregulated by oncogenic signals (4, 28, 29). p19Arf stabilizes p53, which can lead to cell cycle arrest or apoptosis (6, 9, 13). Our data indicate that the p19Arf promoter became activated in some PASMC by day 10 in culture, but in most PASMC by day 42. Normally, these cells would undergo arrest or apoptosis, but in the absence of p19Arf, they continued to proliferate. We examined whether the p19Arf promoter remained activated in the daughter cells of these GFP-positive PASMC.

![Figure 1](http://example.com/image1.png)

**Fig. 1.** p19Arf-deficient pulmonary artery smooth muscle cells (PASMC) demonstrate increased proliferation in culture. Extralobar pulmonary arteries were dissected free from mice, digested, and plated. A: every 7 days, cells from each genotype were counted and then replated at 1 x 10^5 cells/well. Sum of all cells up to, and inclusive of, each time point is shown. Values are means ± SE from 3 mice for each genotype, each cultured separately. *P < 0.05 for p19Arf^+/-. #P < 0.05 for p19Arf^+/-. B: quantitative RT-PCR for p19Arf mRNA in PASMC from each genotype. *P < 0.05 vs. p19Arf^+/+. #P < 0.05 vs. p19Arf^+/-. C: PASMC stained with calcein-AM at 7, 21, and 42 days. Calcein-AM, a cell-permeable stain, is hydrolyzed to calcein, a dye that fluoresces at 598 nm, in live cells.
PASMC, that is, whether some signal induced an epigenetic change within these cells that persisted in daughter cells. To determine this, we used fluorescence-activated cell sorting (FACS) to separate p19\textsuperscript{Arf}-deficient PASMC into GFP-positive and -negative cells and grew them independently. We then used flow cytometry to determine the percentage of GFP-positive cells over 28 days. Figure 5A demonstrates that all initially GFP-positive cells remained GFP-positive over 28 days, indicating that once the p19Arf promoter was activated in a PASMC, the promoter stayed active, not only in that cell, but in all daughter cells. In contrast, GFP-negative cells produced a mixture of GFP-negative and -positive cells over time. By day 17, ~50% of cells were GFP-positive, and by day 28, ~80% of cells were GFP-positive. We did not detect a difference in growth rates between GFP-positive and -negative cells and grew them independently. Western blotting and real-time RT-PCR were then used to determine p19Arf expression in each subset. We found 10-fold greater Bmi-1 expression in GFP-negative than -positive cells. Values are means ± SE of 2 separate experiments. *P < 0.05.

DISCUSSION

The model used in this study, in which the p19\textsuperscript{Arf} gene was not just deleted, but also replaced with GFP, allowed us to examine the timing and cell-specific expression of p19\textsuperscript{Arf} activation and the growth behavior of PASMC in culture. We observed that 1) the loss of p19\textsuperscript{Arf} provided a proliferative advantage to PASMC in culture, primarily due to the failure of p19\textsuperscript{Arf}-deficient cells to arrest or undergo cell death, 2) over time most, but not all, PASMC activated the p19\textsuperscript{Arf} promoter within cell culture, 3) once the p19\textsuperscript{Arf} promoter was activated within a PASMC, it remained activated in all subsequent daughter cells, indicative of an epigenetic imprint, and 4) there was a population of PASMC that did not activate the p19\textsuperscript{Arf} promoter, possibly because of increased Bmi-1 levels.

The Ink4a/Arf locus that encodes p16\textsuperscript{Ink4a} and p19\textsuperscript{Arf} is among the most frequent sites of genetic loss in human cancer (8, 22, 23). Deletion of this locus is seen in a variety of malignancies, including glioblastoma, melanoma, pancreatic adenocarcinoma, and non-small cell lung cancer. Initially, two suppressor genes, p15\textsuperscript{Ink4b} and p16\textsuperscript{Ink4a}, were identified within this locus. These two genes, which encode proteins with a high...
degree of conservation and were thought to arise from gene duplication, enhance the growth-suppressive functions of retinoblastoma by inhibiting cdk4/cdk6 kinase activity (3, 22, 23). Loss of p15 and p16 was initially thought to account for all the increased incidence of malignancy due to mutations at this site on human chromosome 9. In 1997, however, Kamijo and colleagues (7) discovered an alternative first exon (1\textsubscript{H9252}) that had its own promoter but spliced into the second and third promoters shared with p16\textsubscript{Ink4a}, giving rise to p14\textsubscript{ARF} in humans and p19\textsubscript{Arf} in mice. The p16\textsubscript{Ink4a} and p19\textsubscript{Arf} proteins were encoded in alternative reading frames and had no significant amino acid homology. Evidence that p19\textsubscript{Arf} possessed tumor-suppressive activity came from the observation that deleting exon 1\textsubscript{H9252} (while leaving exons 2 and 3 and, thus, p16\textsubscript{Ink4a} intact) mimicked the increased susceptibility to spontaneous and induced tumors seen with the previous exon 2- and 3-deleted mice (14, 22, 24).

p19\textsubscript{Arf} is not usually detected during normal cell growth but is upregulated by oncogenic stimulation, such as mutated Ras and increased c-Myc and E2F expression (14). Expression of p19\textsubscript{Arf} is also increased in murine embryonic fibroblasts grown in culture, presumably in response to the foreign conditions of cell culture (21, 25). Consistent with those observations, we demonstrated that few PASMC expressed p19\textsubscript{Arf} when first plated in culture and showed that p19\textsubscript{Arf}-deficient and wild-type PASMC grew similarly and formed identical-sized colonies at day 10. After day 10, however, the growth curves and the number and size of colonies began to differ, with p19\textsubscript{Arf}-deficient cells claiming a proliferative advantage. This time course correlated with activation of the p19\textsubscript{Arf} promoter in the p19\textsubscript{Arf}-deficient PASMC, indicating that these (GFP-positive) cells had been targeted for cell arrest or cell death by some intrinsic or extrinsic signal but survived and continued to proliferate because of the absence of the p19\textsubscript{Arf} gene.

Not all PASMC activated the p19\textsubscript{Arf} promoter, however, even though all were exposed to the same environmental conditions. By day 42, ~80% of cells were GFP-positive, while 20% remained GFP-negative, indicating that the p19\textsubscript{Arf} promoter had not been activated in these cells. We examined this issue more closely by subcloning the cells on the basis of GFP positivity (i.e., whether they had activated the p19\textsubscript{Arf} promoter) and followed their growth patterns over 28 days. PASMC in which the p19\textsubscript{Arf} promoter had been activated (i.e., were GFP-positive) gave rise to only GFP-positive daughter cells. In contrast, GFP-negative cells generated a mixture of GFP-positive and -negative daughter cells over time and, by day 28, had reestablished a similar 80% GFP-positive and 20% GFP-negative distribution. This surprising finding suggested that epigenetic changes had occurred within the GFP-positive cells that had targeted these cells and their progeny to cell arrest or apoptosis via the p19\textsubscript{Arf}/p53-mediated pathway. Only the absence of p19\textsubscript{Arf} prevented this outcome. In contrast, the cells that remained GFP-negative (i.e., had not activated the p19\textsubscript{Arf} promoter) retained the ability to generate daughter cells that were both GFP-negative and -positive.

The finding that p19\textsubscript{Arf} activation within PASMC in culture was not uniform highlights a limitation of using Western blot analysis to draw conclusions regarding cell behavior in culture. On the basis of previous reports in mouse embryonic fibroblasts and other cell types (21, 24), the increased expression of p19\textsubscript{Arf} in PASMC growing in culture (and, thus, the development of GFP positivity) was expected. The novel finding was that not all cells activated the p19\textsubscript{Arf} promoter, even during prolonged cell culture exposure. The design of the mouse in which the p19\textsubscript{Arf} gene was replaced with GFP allowed us to determine that a small percentage of cells did not activate the

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**Fig. 5.** A subset of p19\textsubscript{Arf}-deficient PASMC do not activate the p19\textsubscript{Arf} promoter. p19\textsubscript{Arf}-deficient PASMC were sorted into GFP-positive and -negative cells by FACS and subcultured separately. A: change in GFP expression over time in both subsets. x-Axis, GFP intensity; y-axis, number of cells. B: growth curves over 7 days in GFP-negative and -positive p19\textsubscript{Arf}-deficient PASMC indicating similar growth patterns. Values are means ± SE of 3 separate experiments.

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**Fig. 6.** Increased Bmi-1 mRNA in GFP-negative p19\textsubscript{Arf}-deficient PASMC compared with GFP-positive cells. p19\textsubscript{Arf}-deficient and heterozygous PASMC were sorted by flow cytometry, and Bmi-1 mRNA was determined using quantitative RT-PCR (qRT-PCR). Results were normalized to Bmi-1 levels in GFP-negative cells. Similar results were obtained with 2 distinct sets of primers. Values are means ± SE of 2 separate experiments. *P < 0.05.
p19<sup>Arf</sup> promoter, an observation that would have been lost had only cell lysates been examined. Similarly, we were able to establish that cells in which the p19<sup>Arf</sup> promoter was not activated (GFP-negative cells) possessed the capacity to generate cells in which the p19<sup>Arf</sup> promoter was either inactive (GFP-negative) or active (GFP-positive), whereas cells in which the p19<sup>Arf</sup> promoter had been activated (GFP-positive cells) could only generate cells in which the p19<sup>Arf</sup> promoter was active. It is reasonable to assume that a similar heterogeneous activation of the p19<sup>Arf</sup> promoter occurs in wild-type cells as well.

Unanswered by this study is the nature of the signal that regulates p19<sup>Arf</sup> promoter activation within these PASMC and the reason that signal is integrated differently within seemingly similar cells growing in the same environment. We did demonstrate an increased expression of the polycomb transcriptional repressor Bmi-1 in cells in which the p19<sup>Arf</sup> promoter was not activated. This observation is consistent with reports of the inhibitory role of Bmi-1 in p19<sup>Arf</sup> expression in hematopoietic (11, 19) and neural (16, 17) resident stem cells. For this study, however, we did not further characterize these cells in terms of putative stem cell surface markers or behavior. These results do provide evidence, however, that the tumor suppressor p19<sup>Arf</sup> is heterogeneously activated within a population of PASMC. Whether the subpopulation of cells that failed to activate the p19<sup>Arf</sup> promoter is descended from cells originally present within the pulmonary arterial wall or developed within the cell culture environment is unclear and requires additional studies.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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