Inhibition of Colon Tumor Progression and Angiogenesis by the Ink4a/Arf Locus


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Abstract

The Ink4a/Arf locus is frequently methylated in colon carcinoma and other common human cancers, suggesting that the locus may play a broad, as yet poorly defined, role inhibiting tumor progression. We examined the influence of the locus in mice with multiple intestinal neoplasia (Min). Colon tumors in 3-month-old Min mice that were null for the Ink4a/Arf locus (−/−) were moderately larger than in Ink4a/Arf-wild-type (+/+), animals (P = 0.032). More strikingly, one-half of the −/− colon tumors were grossly red in color, whereas most of the +/+ tumors were white (P = 0.0025). This color difference remained statistically significant after normalizing for tumor area (P = 0.016). On histological analysis, −/− colon tumors displayed more RBCs on the tumor surface, twice the number of functional vessels, and features of carcinoma in situ not found in +/+ tumors. Biochemical analyses showed that red tumors had higher hemoglobin and vascular endothelial growth factor (VEGF) content than white tumors. Surprisingly, the small intestinal tumor burden was actually lower in −/− animals, and none of these tumors were red, underscoring the importance of tissue context in the function of the locus. These results provide direct evidence that the Ink4a/Arf locus inhibits colon tumor progression. The enhanced vascularity of the −/− tumors is particularly significant in light of the clinical importance of this property in the detection, recurrence, and therapy of colon tumors.

Introduction

The Ink4a/Arf locus encodes the tumor suppressors p16

\(^{Ink4a}\) (p16) and p19

\(^{Arf}\) (Arf) (1). This locus is often methylated in a broad range of common human solid tumors, including carcinomas of the colon and breast (2–4). A considerable body of circumstantial evidence supports the notion that such methylation is relatively specific and results in functionally significant gene inactivation (2). Nonetheless, direct evidence that the locus inhibits tumor progression has been lacking for most tumor types, and the net impact of the locus on progression of these tumors has remained unknown.

p16 binds specifically to Cdns 4 and 6, activates proteins of the pRb family, and arrests cells in G, phase of the cell division cycle. Arf acts through p53 and additional targets to foster cell cycle arrest and apoptosis (1, 5). Thus, both p16 and Arf are viewed as functioning cell-autonomously. However, the role of these proteins in physiological settings of tumor suppression remains poorly understood. There has been no evidence that endogenous p16 and Arf inhibit tumor angiogenesis, but exogenous expression of p16, Arf, and some elements of their response pathways exerts antiangiogenic effects (6–9).

Colon carcinoma, the second most common fatal human malignancy, has served as a prototype for studies of tumor progression (10). Colon carcinomas generally develop from benign adenomas, which can be readily identified and removed, providing tissue for analysis. Methylation of the p16 and/or Arf promoters has been found in about one-half of colon carcinomas and adenomas, suggesting that the locus may suppress early stages of development of this tumor type (3, 4). Consistent with this notion, p16 expression has been found in subsets of cells within colon adenomas, in which it correlates with cell cycle arrest (11). Recently, vascularity has been recognized as a clinically important feature of colon tumor progression. Detection of bleeding into the lumen provides a key screening method for colon cancer (12). Furthermore, the extent of vascularity within primary tumors helps to predict the likelihood of recurrence after therapy (13). Finally, evidence has emerged that tumor vasculature is a key target of nonsteroidal anti-inflammatory drugs effective in the treatment and prophylaxis of colon tumors (14). Nonetheless, the molecular events that regulate angiogenesis in colon tumors are not well understood.

The availability of excellent mouse models of colon neoplasia permits genetic analysis of the influence of the Ink4a/Arf locus on the disease. The Min strain is the best characterized of such models (15). Min mice harbor a mutation in the adenomatous polyposis coli gene, the gene most commonly inactivated in human colon neoplasia. Min mice develop intestinal adenomas by 3 months of age. Small intestinal tumors are more numerous, but the colon tumors are larger (typically more than 4 × the area per tumor). Although p16 and Arf expression have not been described in Min adenomas, p16 is expressed in mutagen-induced mouse colon adenomas (16). As in human colon adenomas (11), p16 expression is heterogeneous and inversely correlated with pRb expression (16), likely reflecting down-regulation of pRb after p16-mediated arrest (11, 17, 18). When Min mice were bred to mice lacking p53 in a uniform genetic background, a modest increase in average adenoma size was observed, with an increase in advanced histological features of borderline statistical significance (19). A mouse strain has been derived in which the second and third exons of the Ink4a/Arf locus are deleted, abrogating the function of both p16 and Arf (1, 20). Mice homozygous for this deletion (−/−) have no known intestinal phenotype but begin to die of sarcomas and lymphomas by 6 months of age. By breeding the Ink4a/Arf −/− mutation into a Min background, we assessed the influence of the Ink4a/Arf locus on colon tumor progression.

Materials and Methods

Genetic Models and Genotyping. Min mice were obtained in a pure C57Bl/6 genetic background. Ink4a/Arf −/− mice were originally of a mixed

Received 9/11/02; accepted 12/27/02.

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1 Supported by a Foundation for Digestive Health and Nutrition Miller and Shirley Fiterman Basic Research grant (to G. H. E.), an American Cancer Society Research Scholar Grant RPG-99-168-01-CCG (to G. H. E.), a pilot project grant from the NIH Center for Molecular Studies in Digestive and Liver Diseases at the University of Pennsylvania (to G. H. E.), a NIH individual National Research Service Awards (to S. L. G.), a NIH Cell and Molecular Biology training grant (to C. Y. D.), and the NIH Medical Scientist Training Program (S. L. G., C. Y. D., and M. S. G.).

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4 The abbreviations used are: Arf, alternative reading frame; Cdk, cyclin-dependent kinase; Rh, retinoblastoma. Min, multiple intestinal neoplasia; TAU, Triton X-100-acetic acid-urea (gel); VEGF, vascular endothelial growth factor; Hb, hemoglobin.
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C57Bl/6:129Sv background (20). C57Bl/6 and 129Sv strains share the same genotype for the major modifier of Min tumor formation (15). Nonetheless, the Ink4a/Arf−/− mice were backcrossed for seven generations into the C57Bl/6 background, to reduce the likelihood of asymmetric segregation of minor modifiers derived from 129Sv sequences. All of the mice scored for tumor formation, including Ink4a/Arf+/− mice, were derived from Min matings with the backcrossed Ink4a/Arf−/− strain. Genotypes were determined by PCR from animal tail DNA prepared by Proteinase K digestion. The Min genotype was assessed as described previously (21). For determining the Ink4a/Arf genotype, two separate PCR reactions were used. The first primer set, 5’-ATGATGATGGGCAAGTTCAC-3’ and 5’-CGTTGTTGACTGAACTGAT-3’, amplified exon 2 of the wild-type locus, and the second set, 5’-TGAAAAGTGATTGGAGCGC-3’ and 5’-GTCAGAAGGCGATAAAGGCCG-3’, amplified the neomycin resistance gene of the “knockout” mutation. The following PCR program was used for all of the reactions: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, repeated for 40 cycles. All of the products were resolved by electrophoresis through a 3% agarose gel.

Tumor Histology. Formalin-fixed, paraffin-embedded tumors were sectioned and stained with H&E. A pathologist (E.E.F.) blinded to the genotypes examined at ×10, sections that approximately bisected the tumor. Cribriforming was identified by the presence of sheets of neoplastic epithelium forming honeycomb patterns of interlocking bands (22). Pockets of necrosis were identified as round-to-oval areas of open space many cell widths in diameter, partially filled, with cellular debris and inflammatory cells (23). Sections were scored for the number of RBCs in areas of highest apparent RBC density outside the tumor stalk, at ×20. RBCs were classified as being either within a visible blood vessel or within the interstitial space (no vessel apparent).

Confocal Microscopy and Blood Vessel Quantitation. Ink4a/Arf−/− and +/+ 3-month-old Min mice were injected through the major tail vein 10–20 min prior to sacrifice with a fluorescein isothiocyanate- and rhodamine-conjugated tomato lectin (Vector) that labels endothelial cells (24). Colon tumors were resected, cut into 1-mm-thick slices, and mounted on slides in cold PBS. Images from areas of highest microvascular density (25) were acquired by confocal microscopy at ×10 with a fluorescein filter. Stacks of images of up to 24 discrete focal planes were merged (24). Midline horizontal and vertical bisector lines were superimposed over each composite image, and vessels crossing these lines were scored at the point of intersection.

Immunoblotting and ELISA. Colon tumor lysates were prepared as described previously (11). Colon mucosa was scraped from the luminal surface with a razor blade. The specificity of the mucosal preparations was confirmed by H&E staining of formalin-fixed, paraffin-embedded sections. Samples were diluted in E1A lysis buffer (11) and subjected to electrophoresis through standard polyacrylamide gels (SDS-PAGE) or through TAU-PAGE (26). Membranes were probed with a rabbit anti-Hb antibody (ICN; Kappel). VEGF levels in 10 µg of protein extract were assayed by ELISA (R&D Systems), using a purified VEGF standard.

Statistical Methods. Generalized estimating equations were used to compare the area per tumor between genotypes (27). Because the distribution of area per tumor was right-skewed, the square root transformation was used to obtain an approximately normal distribution. Two-sample t tests were used to compare between genotypes the total tumor area, number of tumors, and number of RBCs within and outside of vessels. A generalized estimating equation logistic regression analysis was used to assess the red coloration between the genotypes, with and without adjusting for tumor area. The Fisher’s exact test was used to compare the frequency of cribriforming and necrosis between genotypes. The Wilcoxon rank-sum test was applied to the number of lectin-stained blood vessels per bisector. In each case in which two sample t tests or Wilcoxon rank sum tests were used, the other method was also used, with similar results.

Results

Accelerated Progression of Ink4a/Arf−/− Colon Tumors. We compared the size and histological features of colon tumors from Ink4a/Arf−/− and +/+ Min mice in a C57Bl/6 genetic background. We sacrificed mice at 3 months of age, at which point tumors can be detected on gross inspection of the colon in most mice, but there is little genotype-related morbidity or mortality. From 29 Ink4a/Arf−/− and 25 Ink4a/Arf+/+ Min mice, we obtained 45 and 33 colon tumors, respectively (P = 0.42; Table 1; Fig. 1, A–C). Ink4a/Arf−/− colon tumors were moderately larger than the corresponding Ink4a/Arf−/+ tumors (1.5X, P = 0.032; Table 1). Colon tumor burden, defined as the total tumor area per animal, was also greater in Ink4a/Arf−/− mice (1.8X, P = 0.031).

For histological analysis, six Ink4a/Arf−/− and six +/+ colon tumors were fixed in formalin, embedded in paraffin, and stained with H&E. Small Ink4a/Arf−/− tumors (those less than the mean tumor size) were excluded. The largest Ink4a/Arf−/+ tumors were selected, including two from older mice (4 and 6 months), to match the size of Ink4a/Arf−/− tumors (Table 1). Sections approximately bisecting the tumor were presented in random order to a pathologist (E.E.F.) blinded to the genotypes. Each of the Ink4a/Arf−/− tumors showed substantial histological features of carcinoma in situ, manifested by both cribriform neoplastic epithelium (Ref. 22; Fig. 2, A and C; white arrows) and pockets of necrosis (Ref. 23; Fig. 2, A and C; black arrows), without invasion of the submucosa. Conversely, none of the Ink4a/Arf−/+ tumors had substantial cribriforming or necrosis (Fig. 2, B and D; P = 0.0022; Table 1).

In initial experiments, we noted that Ink4a/Arf−/− tumors were often pink or red (“red”), whereas the Ink4a/Arf+/+ tumors were typically white (Fig. 1, A and B). We, therefore, scored this parameter on the complete set of tumors. Twenty-two (49%) Ink4a/Arf−/− mice showed histological features of adenocarcinoma in situ, whereas none of the Ink4a/Arf+/+ tumors were “red” (P = 0.0022; Table 1).

Table 1 Features of accelerated colon tumor progression in Ink4a/Arf−/− mice

<table>
<thead>
<tr>
<th>Ink4a/Arf genotype</th>
<th>−/−</th>
<th>+/+</th>
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<tbody>
<tr>
<td>Tumor size</td>
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<tr>
<td>Tumors identified</td>
<td>45</td>
<td>33</td>
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<tr>
<td>Mean area</td>
<td>12 ± 7.5</td>
<td>7.7 ± 6.8</td>
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<tr>
<td>Fold size difference</td>
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<td>0.032</td>
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<tr>
<td>Tumor burden</td>
<td></td>
<td></td>
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<tr>
<td>Mice examined</td>
<td>29</td>
<td>25</td>
</tr>
<tr>
<td>Mean tumor burden/mouse</td>
<td>18 ± 14</td>
<td>10 ± 11</td>
</tr>
<tr>
<td>Fold burden difference</td>
<td>1.8 ×</td>
<td>0.031</td>
</tr>
<tr>
<td>Tumor color</td>
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<td></td>
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<tr>
<td>Tumors examined</td>
<td>45</td>
<td>33</td>
</tr>
<tr>
<td>Red tumors</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>% red tumors</td>
<td>49</td>
<td>12</td>
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<tr>
<td>Odds ratio</td>
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<td>0.0022</td>
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<tr>
<td>Odds ratio, area-adjusted</td>
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<td>0.016</td>
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<tr>
<td>Histological features</td>
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<tr>
<td>Tumors examined</td>
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<td>6</td>
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<tr>
<td>Mean area</td>
<td>19.8 ± 5.2</td>
<td>17.5 ± 2.3</td>
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<tr>
<td>P</td>
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<tr>
<td>Cribriforming and necrosis</td>
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<tr>
<td>P</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td>Mean no. RBCs within vessels</td>
<td>27 ± 11</td>
<td>6 ± 5</td>
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<tr>
<td>P</td>
<td>0.0022</td>
<td></td>
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<tr>
<td>Mean no. RBCs outside vessels</td>
<td>61 ± 32</td>
<td>9 ± 3</td>
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<tr>
<td>P</td>
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<td>Tumor vessels, lectin-stained</td>
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<tr>
<td>Tumors examined</td>
<td>6</td>
<td>4</td>
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<tr>
<td>Mean no. vessels</td>
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<td>4.1 ± 2.3</td>
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<tr>
<td>P</td>
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<tr>
<td>Mean diameter</td>
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<tr>
<td>P</td>
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<tr>
<td>Mean distance to branch point</td>
<td>5.3 ± 1.2</td>
<td>8.5 ± 5.1</td>
</tr>
<tr>
<td>P</td>
<td>0.17</td>
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</table>
underscoring the importance of tissue context in function of the locus.

hypothesized that the increased red coloration in Ink4a/Arf

regression analysis of the data to adjust for the size effect. After

wide range of sizes (Fig. 1). We, therefore, performed a logistic


tumors were red versus 4 (12%) Ink4a/Arf +/+ tumors (P = 0.0025;

Fig. 1C; Table 1). Although we noted a correlation between tumor

and red coloration (P = 0.0011), red tumors were found over a

wide range of sizes (Fig. 1C). We, therefore, performed a logistic

regression analysis of the data to adjust for the size effect. After

normalizing for size, Ink4a/Arf −/− tumors still were more often red

(P = 0.016, Table 1).

Surprisingly, small-intestinal-tumor burden was actually lower

(0.59 times) in Ink4a/Arf −/− animals (n = 13) than +/+ animals

(n = 12; P = 0.016), and none (0/1469) of these tumors were red,

underscoring the importance of tissue context in function of the locus.

Increased Vascularity of Ink4a/Arf −/− Colon Tumors. We

hypothesized that the increased red coloration in Ink4a/Arf −/− colon

tumors was due to an increased vessel density and blood content.

Before sacrifice, we injected mice i.v. with a fluorescein-conjugated

lectin that labels the lumenal surface of functional vessels (24). We

examined tumors by confocal microscopy and scored blood vessels in

images obtained from areas of highest microvessel density (Ref. 25;

Fig. 1, D and E). We scored the number of vessels that crossed bisecting lines superimposed on the images, vessel diameter at these

points, and the distance to the nearest vessel branch point. Ink4a/Arf

−/− tumors demonstrated twice the number of blood vessels

(P = 0.043; Table 1). A trend was noted in Ink4a/Arf −/− tumors

toward narrower blood vessel diameter and greater vessel branching

(shorter distance to branch points; Table 1).

Increased RBC Density in Ink4a/Arf −/− Colon Tumors. We

sought to confirm by histological and biochemical analyses that the

red coloration of tumors reflected an increased blood content. Histological analysis of H&E-stained sections allowed us to visualize individual RBCs (Fig. 2, E–H). Large pools of RBCs were not seen, arguing against major episodes of intratumoral hemorrhage. However, Ink4a/Arf −/− tumors had areas with high RBC density near the luminal surface of the colon, distant from the occasional large vessels in tumor stalks. These RBCs were present both within vessel-like structures (Fig. 2, E and G; long, thin arrows) and within interstitial spaces, with no vessels apparent (Fig. 2, E and G; short, thick arrows).

![Fig. 1. Increased red coloration and functional vessels in Ink4a/Arf −/− colon tumors. A and B, Min mice were sacrificed at 3 months of age. The colons were resected and opened to expose the lumenal surface. Methylene blue (1%) in PBS was applied, to provide contrast between the elevated tumor and the surrounding normal mucosa. Tumors were measured at their greatest diameter and photographed through a dissecting microscope (×6). Representative images from Ink4a/Arf −/− (A) and Ink-4a +/+ (B) mice are shown. C, tumor sizes were graphed (Ink4a/Arf −/− on left; Ink4a/Arf +/+ on right), with area on the y-axis. D and E, mice were injected i.v. with a fluorescein-conjugated lectin that adheres to functional vessel lumens. Colon tumors were resected and examined by confocal microscopy. Areas of greatest microvessel density were photographed. Representative ×10 images are shown (D: Ink4a/Arf −/−; E: Ink4a/Arf +/+).](image1)

![Fig. 2. Advanced histological features and increased RBC content in Ink4a/Arf −/− colon tumors. Colon tumors were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E. A–D, representative ×10 images demonstrate findings of substantial cribriforming of neoplastic epithelium (white arrows) and pockets of necrosis (black arrows) in Ink4a/Arf −/− tumors (A, C). E–H, representative ×20 images near the surface of tumors demonstrate increased RBC content in Ink4a/Arf −/− tumors (E, G) both within apparent vessels (long, thin red arrows) and outside such structures (short, thick red arrows).](image2)
By contrast, RBCs were rare near the surface of Ink4a/Arf+/+ tumors, either within or outside of vessel-like structures (P = 0.0022 and 0.0010, respectively; Fig. 2, F and H; Table 1).

**Increased Hb and VEGF Content in Red Tumors.** We also assayed total Hb content of tumors by immunoblotting. Five Ink4a/Arf−/− and 5 +/+ Min colon tumors from 3-month-old mice were assayed, of different colors and overlapping masses (Fig. 3A; data not shown). Two electrophoresis methods were used. In standard SDS-PAGE, the α and β globins of Hb comigrate. In TAU-PAGE (26), most proteins are excluded from the gel by their net negative charge, and α and β globins can be resolved. To detect Hb, we used an antibody that can detect both globins but is more sensitive for β globin. We prepared extracts from blood and lung tissues as positive controls and extracts from cultured mouse embryo fibroblasts as negative controls. By both electrophoretic methods, red tumors displayed greater Hb content than genotypically matched white tumors (Fig. 3A, data not shown). The red tumors also had greater Hb content than neighboring intact colon or colonic mucosa, which were grossly white. Similar results were obtained using antibodies specific for α and β globins (gift of Mitch Weiss, University of Pennsylvania, Philadelphia, PA; data not shown). In initial studies of angiogenic signaling, we assayed the VEGF content of the tumor extracts. Red tumors had higher VEGF levels than white tumors or normal colon (Fig. 3B).

**Discussion**

Colon carcinoma is important both as a commonly fatal disease and as a tractable model for the study of tumor progression (10). A considerable body of circumstantial evidence suggests that the Ink4a/Arf locus suppresses colon carcinoma, but direct evidence has been lacking. Moreover, the specific steps of colon tumor progression countermanded by the locus and the net impact of the locus on tumor progression have remained unknown. We present evidence that Min colon tumors progress more rapidly in the absence of p16 and Arf. This result establishes that the locus inhibits progression of a solid tumor initiated by an independent genetic event. A broad role for the Ink4a/Arf locus as an inhibitor of tumor progression is also suggested by the early observation that the locus undergoes deletion in advanced stages of some experimental mouse skin tumors (28) and the recent observation that advanced lung tumors appear to be more common in mutagen-treated mice that lack p16 (29). Moreover, a striking and largely unanticipated finding reported here is that Ink4a/Arf−/− colon tumors were more vascular, an effect partially independent of tumor size. The vascular phenotype, suggested by gross inspection, was verified by four different histological and biochemical methods in a total of 32 tumors. This phenotype is particularly noteworthy because vascularity is recognized as a clinically important property of colon tumors.

The increased vascularity of Ink4a/Arf−/− colon tumors appears to result, at least in part, from an increase in blood vessel number. These vessels appear to be primarily small-caliber vessels at the periphery of tumors, based on findings from standard histology and lectin staining of functional vessels. Such vessels have been seen previously in studies of colon tumors (30) and are thought to be formed by angiogenesis rather than by dilation of stalk vessels, coopting of existing mucosal vessels, or formation of pseudo-vessels by neoplastic epithelium (31). These findings are consistent with the increased VEGF content observed in red tumors in our initial studies of angiogenic signaling.

The effect on tumor vascularity is somewhat surprising because of abundant in vitro evidence that p16 and Arf act cell-autonomously to inhibit proliferation of neoplastic cells (1). Our results provide the first evidence that endogenous p16 and Arf inhibit tumor angiogenesis. One possibility is that these proteins actively inhibit angiogenic signaling from the neoplastic epithelium. Consistent with this notion, a recent study suggests that Arf may be required during embryonic development for regression of a vascular structure in the mouse eye (32). In human colon tumors, p16 expression was often found in neoplastic epithelial cells bordering normal tissue (11), a position well suited to influence angiogenic signaling. Further work will be required to determine how direct or indirect is the effect of the Ink4a/Arf locus on colon tumor vascularity.

The function of the Ink4a/Arf locus in tumor suppression appears to depend on tissue context, a notion underscored by the lower small intestinal tumor burden in Ink4a/Arf−/− mice compared with +/+ mice and the absence of red tumors in the small intestine. The larger size of colon tumors may contribute to the vascular phenotype evident in tumors from this tissue. More than one-half of colon tumors had diameters greater than 3.8 mm, whereas fewer than 20% of small intestinal tumors had diameters greater than 1.9 mm. An angiogenic “switch” appears to be necessary for the continued growth of a
number of solid tumors beyond 1–3 mm in diameter (33). Nonetheless, the reason for the overall lower small-intestine-tumor burden in Ink4a/Arf−/− compared with +/+ mice remains an enigma.

In summary, we have demonstrated that the Ink4a/Arf locus inhibits colon tumor progression in Min mice. These results add direct functional data to the existing circumstantial evidence that p16 and Arf suppress colon neoplasia. The results support the notion that the frequent methylation of the locus in tumors of the colon and other organs counteracts a broad tumor suppressor role. By contrast, the absence of a tumor suppressor phenotype in the small intestine of Min mice may provide clues to tissue-specific and stage-specific effects of the locus on tumor development. The results also implicate the Ink4a/Arf locus as an important determinant of colon tumor vascularity. Further study using this model system will likely reveal additional such determinants that are affected by this locus.

Acknowledgments

We thank Dr. Mitch Weiss for his help in analysis of Hb and Dr. Anil Rustgi for comments on the manuscript. We thank the Morphology Core Facility of Penn’s NIH/National Institute of Diabetes and Digestive and Kidney Diseases Center for Molecular Studies in Digestive and Liver Disease (P30 DK50306) for assistance.

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