

p21^{Cip1} Nullizyosity Increases Tumor Metastasis in Irradiated Mice¹

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Abstract

p21^{Cip1} is a cyclin-dependent kinase inhibitor whose abundance increases in cells exposed to radiation or other DNA-damaging agents. Such increases activate a G1 checkpoint, which allows time for DNA repair before S phase entry. By inhibiting cell cycle progression, p21^{Cip1} potentially suppresses tumorigenesis, and in support, we show that p21^{Cip1} heterozygous and nullizygous mice develop more tumors than do wild-type mice when exposed to a single dose of γ -irradiation. Importantly, we also show that p21^{Cip1} nullizyosity increases the incidence of metastatic tumors in irradiated mice. We suggest that p21^{Cip1} is haploinsufficient for tumor suppression and functions as an antimetastatic agent.

Introduction

Although the p21^{Cip1} gene is rarely mutated in human tumors (1), p21^{Cip1} is expressed at subnormal amounts or is dysfunctional in some human tumors. Cancer-related events that reduce p21^{Cip1} expression include the functional inactivation of the tumor suppressor and transcription factor p53, an event that occurs frequently in human tumors (2–4), and the hypermethylation and consequent silencing of the p21^{Cip1} promoter, which occurs in leukemic bone marrow cells (5). The *Her-2/neu* gene is often amplified in breast cancers (6), and events downstream of *Her-2/neu* may limit the accessibility of p21^{Cip1} to cyclin-dependent kinases (7). Importantly, inefficient expression of p21^{Cip1} is indicative of short survival times for patients with acute lymphoblastic leukemia or colorectal cancer (5, 8). Although p21^{Cip1}-null mice do not spontaneously develop tumors, at least ≤ 1.5 years of age (9, 10), they are often more susceptible to tumor formation initiated by oncoproteins, carcinogens, or loss of tumor suppressors (11–14). However, the role of p21^{Cip1} in tumor suppression remains unclear (15–17), and our studies examined in detail the effects of p21^{Cip1} gene dosage on tumor formation in irradiated mice. We show that p21^{Cip1} heterozygosity and nullizyosity increase tumor incidence in irradiated mice, whereas p21^{Cip1} nullizyosity (but not heterozygosity) increases tumor metastasis.

Materials and Methods

Mice and Treatment. Mice with inactivating targeted mutations of both p21^{Cip1} alleles (obtained from Dr. Tyler Jacks, Massachusetts Institute of Technology; Ref. 18) were crossed with wild-type mice (The Jackson Laboratory, Bar Harbor, ME), which have a comparable mixed C57BL/6J \times 129S1/SvImJ (B6;129S) genetic background. p21^{+/-} progeny were sibling mated to produce p21^{+/+}, p21^{+/-}, and p21^{-/-} mice. Genotyping was performed as

described previously (11). At 14 ± 2 days of age, mice received a single 4-Gy dose of whole body γ -irradiation from a ¹³⁷Cs source. After irradiation, mice were monitored daily by visual inspection and sacrificed when tumors of ≥ 1 cm were noted, when morbidity was noted, or at 14 months of age. Euthanasia was performed in accordance with the procedures outlined in the 2000 Report of the American Veterinary Medical Association Panel on Euthanasia. All experimental and surgical procedures performed on mice were in accordance with the guidelines of the University of South Florida and the NIH guidelines outlined in the “Guide for Care and Use of Laboratory Animals” (NIH publication 85-23).

Histological Analysis. Necropsies were done in the following stepwise manner. The skin, subcutis, skeletal muscle, inguinal lymph node and mammary fat pad, cervical lymph node, and salivary glands were inspected visually for tumors or other abnormalities and collected. The entire alimentary tract, including the esophagus, stomach, small intestine, cecum, and colon, was inflated with 10% neutral-buffered formalin and rolled in segments to fit into cassettes for histological processing. The reproductive tract and associated glands and organs, urinary bladder, spleen, pancreas, kidneys, adrenal glands, liver, and gall bladder were evaluated and collected. The larynx, trachea with attached thyroid and parathyroid glands, and lungs were inflated with 10% neutral-buffered formalin, fixed with the heart and thymus, and trimmed and placed in cassettes for processing. The brain cut in three cross-sections, portions of the spinal column in lateral and longitudinal sections, pituitary gland, eyes, and Harderian gland were inspected and collected. Tumors and tissues were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, sectioned at 3 μ m, and stained with H&E. Histological sections were masked and interpreted independently by R. W. E. and D. C. Discrepancies were resolved by consultation with other pathologists. Over 3200 histological preparations were examined. Staining of selected tumor sections for p21^{Cip1} expression was done as described previously (13).

Statistical Analysis. Statistical comparisons of proportions in all three groups and between pairs of groups were based on Fisher’s exact test. Numbers of tumors per animal were compared using the Kruskal-Wallis Test, a non-parametric analogue of the pooled *t* test, and one-way ANOVA. Survival rates over time were compared using the Log-rank test. All reported *P*s are two sided. All statistical analyses were performed using SAS (version 8.2). *P*s < 0.05 are considered significant, and *P*s between 0.05 and 0.1 are considered suggestive.

Results and Discussion

Approximately 200 mice (p21^{+/+}, p21^{+/-}, and p21^{-/-} genotypes) received a 4-Gy dose of whole body γ -irradiation at 2 weeks of age. Irradiated mice were monitored daily and necropsied when signs of illness or palpable tumors were seen. Mice that did not develop overt symptoms of disease were considered “symptom free” and necropsied 13.5 months after γ -irradiation. Necropsies were done on 31 p21^{+/+} mice, 105 p21^{+/-} mice, and 61 p21^{-/-} mice, and all organs were inspected macroscopically and microscopically. p21^{Cip1} gene dosage had no significant effect on the symptom-free survival of γ -irradiated mice (data not shown). All mice were symptom free at 4 months of age, and 70% of all mice were symptom free at 14 months of age. However, as determined by necropsy and histological analysis, most mice were tumor bearing. The percentages of p21^{+/+}, p21^{+/-}, and p21^{-/-} mice with tumors were 68, 79, and 80%, respectively, and not

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significantly different. On the other hand, p21^{+/-} and p21^{-/-} mice had significantly more tumors per mouse than did p21^{+/+} mice ($P = 0.0277$ in a three-way comparison; Table 1). Average numbers of tumors per mouse were 2.23 for p21^{+/-} mice ($P = 0.016$) and 2.22

for p21^{-/-} mice ($P = 0.01$), as compared with 1.1 for p21^{+/+} mice. Moreover, 52% of p21^{+/-} mice ($P = 0.0032$) and 54% of p21^{-/-} mice ($P = 0.0045$) had more than one tumor per mouse, as compared with only 23% of p21^{+/+} mice. Immunohistochemistry confirmed the

Table 1 Incidence and spectrum of tumor types in γ -irradiated p21^{+/+}, p21^{+/-}, and p21^{-/-} mice

Shaded tumors are malignant. Asterisk (*) denotes tumors of mesenchymal origin. Because seven p21^{+/-} and seven p21^{-/-} mice were not necropsied because of advanced autolysis, the data shown may underestimate the number of tumors in the p21^{+/-} and p21^{-/-} cohorts. The P s comparing the number of tumors per mouse for p21^{+/-} and p21^{-/-} mice with those for p21^{+/+} mice were significant for the Harderian gland and ovary. Harderian gland: p21^{+/-}, $P = 0.0267$; p21^{-/-}, $P = 0.0484$. Ovary: p21^{+/-}, $P = 0.0077$; p21^{-/-}, $P = 0.0052$.

Tumor		Number of tumors (Tumors per mouse)		
Primary organ	Morphological diagnosis	p21 ^{+/+} , 31 mice	p21 ^{+/-} , 105 mice	p21 ^{-/-} , 61 mice
Adrenal gland	Cortical carcinoma	0	2 (0.019)	0
	Pheochromocytoma	0	1 (0.010)	1 (0.016)
Brain	Ependymoma	0	0	1 (0.016)
	Glioblastoma multiforme	0	0	1 (0.016)
Harderian gland	Adenoma	2 (0.065)	20 (0.190)	10 (0.164)
	Adenocarcinoma	0	6 (0.057)	4 (0.066)
Liver	Hepatocellular adenoma	1 (0.032)	5 (0.048)	2 (0.033)
	Hemangioma *	0	0	2 (0.033)
	Hepatocellular carcinoma	0	3 (0.029)	3 (0.049)
	Hepatoblastoma	0	0	1 (0.016)
	Histiocytic sarcoma *	0	0	1 (0.016)
	Undifferentiated sarcoma *	0	1 (0.010)	0
Lung	Bronchioloalveolar adenoma	2 (0.065)	5 (0.048)	6 (0.098)
	Chemodectoma*	0	1 (0.010)	0
	Mesothelioma	0	1 (0.010)	0
	Bronchioloalveolar carcinoma	3 (0.097)	14 (0.133)	3 (0.049)
	Undifferentiated sarcoma *	0	0	1 (0.016)
	Hemangiosarcoma *	0	0	1 (0.016)
Ovary ^a	Tubulostromal adenoma	0	17 (0.354)	12 (0.375)
	Gonadal stromal adenoma	2 (0.133)	10 (0.208)	9 (0.281)
	Granulosa cell tumor	0	4 (0.083)	2 (0.063)
Pancreas	Islet cell adenoma	0	1 (0.010)	2 (0.033)
Pituitary gland	Adenoma pars distalis	1 (0.032)	5 (0.048)	6 (0.098)
	Adenoma pars intermedia	0	0	2 (0.033)
	Adenocarcinoma pars intermedia	0	1 (0.010)	0
Prepuccial gland ^b	Adenoma	0	2 (0.035)	0
	Adenocarcinoma	1 (0.063)	0	0
Salivary gland	Adenoma	0	3 (0.029)	1 (0.016)
	Myoepithelioma	0	0	1 (0.016)
	Hemangiosarcoma *	0	1 (0.010)	0
Seminal vesicle ^b	Adenoma	0	1 (0.010)	0
	Granular cell tumor	0	1 (0.010)	0
Skin	Trichoepithelioma	1 (0.032)	0	0
	Carcinoma, epithelial cell	2 (0.065)	0	1 (0.016)
	Leiomyosarcoma *	0	3 (0.029)	0
	Malignant fibrous histiocytoma *	0	1 (0.010)	0
Small intestine	Adenoma	9 (0.290)	42 (0.400)	16 (0.262)
	Adenocarcinoma	2 (0.065)	19 (0.181)	11 (0.180)
	Leiomyosarcoma *	0	0	1 (0.016)
Spleen	Hemangioma *	0	1 (0.010)	0
	Hemangiosarcoma *	0	0	2 (0.033)
Stomach	Adenoma	0	1 (0.010)	1 (0.016)
Testes ^b	Sertoli cell tumor	0	1 (0.018)	0
	Hemangioma *	0	1 (0.018)	0
Thyroid	Follicular cell adenoma	0	0	1 (0.016)
	c-Cell carcinoma	0	0	1 (0.016)
Undetermined	Osteogenic sarcoma*	0	1 (0.010)	0
	Leiomyosarcoma*	0	1 (0.010)	0
Urethra	Papilloma	0	1 (0.010)	0
Urinary bladder	Transitional cell papilloma	0	0	1 (0.016)
Uterus ^a	Endometrial adenoma	1 (0.067)	1 (0.021)	0
	Leiomyoma *	0	1 (0.021)	1 (0.031)
	Benign sarcoma *	0	2 (0.042)	0
	Endometrial carcinoma	0	1 (0.021)	0
Vagina ^a	Fibroma *	0	0	1 (0.031)
	Lymphoma*	3 (0.097)	5 (0.048)	4 (0.066)
Benign tumors per mouse		0.72	1.56	1.57
Malignant tumors per mouse		0.39	0.67	0.65
Total tumors per mouse		1.11	2.23	2.22

^a For the ovary, uterus, and vagina, tumors per mouse was determined by dividing tumor number by the number of female mice: 15, 48, and 32 for the p21^{+/+}, p21^{+/-}, and p21^{-/-} cohorts, respectively.

^b For the prepuccial gland, seminal vesicle, and testes, tumors per mouse was determined by dividing tumor number by the number of male mice: 16, 57, and 29 for the p21^{+/+}, p21^{+/-}, and p21^{-/-} cohorts, respectively.

presence of p21^{Cip1} in tumors of p21^{+/-} mice (Fig. 1). These findings suggest that the p21^{Cip1} gene is haploinsufficient for tumor suppression in γ -irradiated mice.

Tumors were classified as benign or malignant based on morphology. Tumors were considered benign if they contained few mitotic figures, lacked cellular pleomorphisms, were localized and well demarcated, and consisted of expansible masses of well-differentiated cells. Tumors were considered malignant if they had infiltrating

advancing margins, large areas of necrosis, numerous and often atypical mitotic figures, and pleomorphic cells. p21^{+/-} and p21^{-/-} mice developed 2.2-fold more benign tumors ($P = 0.0299$ in a three-way comparison) and 1.7-fold more malignant tumors per mouse than did p21^{+/+} mice (Table 1). The latter difference was not significant perhaps because of small sample numbers. However, p21^{+/-} mice (11%; $P = 0.0934$) and p21^{-/-} mice (10%; $P = 0.0687$) developed multiple malignant tumors per mouse (in separate organ systems), whereas p21^{+/+} mice did not. These findings show that a greater incidence of benign and perhaps also malignant tumors accounts for the greater incidence of total tumors in p21^{Cip1} heterozygous and nullizygous mice, as compared with wild-type mice.

All three groups of irradiated mice developed tumors in several organs, most notably the Harderian gland, lung, ovary, and small intestine (Table 1). p21^{+/-} and p21^{-/-} mice developed significantly more Harderian gland and ovarian tumors per mouse than did p21^{+/+} mice (see P s in the legend to Table 1). Moreover, the spectrum of tumor types was broader in p21^{+/-} and p21^{-/-} mice than in p21^{+/+} mice. Tumor types present in p21^{+/-} and p21^{-/-} mice but not in p21^{+/+} mice include adrenal gland pheochromocytomas, Harderian gland adenocarcinomas, hepatocellular carcinomas, and ovarian tubulostromal adenomas and granulosa cell tumors. Other than thymic lymphomas, which were present in all three cohorts, p21^{+/+} mice did not develop tumors of mesenchymal cell origin. In contrast, p21^{+/-} and p21^{-/-} mice developed a variety of mesenchymal tumors, including hemangiomas, hemangiosarcomas, and leiomyosarcomas. Thus, loss of one or both copies of the p21^{Cip1} gene increases the incidence of Harderian gland and ovarian tumors and the diversity of benign and malignant tumors in irradiated mice.

Despite similar numbers of malignant tumors per mouse, p21^{Cip1} nullizygous mice developed 2.7-fold more metastatic tumors per mouse than did p21^{Cip1} heterozygous mice (Fig. 2A and Table 2, $P = 0.018$). Numbers of metastatic tumors per mouse were 0.1, 0.09, and 0.24 for p21^{+/+}, p21^{+/-}, and p21^{-/-} mice, respectively ($P = 0.0366$ in a three-way comparison). Thymic lymphomas, which invariably were disseminated, accounted for all of the metastatic tumors in p21^{+/+} mice and >50% of the metastatic tumors in p21^{+/-} mice. On the other hand, 70% of the metastatic tumors in p21^{-/-} mice arose from organs other than the thymus; e.g., the Harderian gland, ovary, and spleen. Examples of distant metastases in p21^{Cip1}-null mice are shown in Fig. 2B–D. When thymic lymphomas were excluded from the analysis, the numbers of metastatic tumors per mouse were 0.18 for p21^{-/-} mice, as compared with zero for p21^{+/+} mice ($P = 0.0078$) and 0.04 for p21^{+/-} mice ($P = 0.0147$; $P = 0.0035$ in a three-way comparison). Moreover, 26% of nonlymphoma malignant tumors in p21^{-/-} mice metastasized as compared with 0% in p21^{+/+} mice and 6% in p21^{+/-} mice ($P = 0.0086$ in a three-way comparison). These findings clearly show that deletion of both copies of the p21^{Cip1} gene promotes tumor metastasis in γ -irradiated mice.

Our study provides the most in-depth analysis to date of the combined effects of p21^{Cip1} gene dosage and radiation on whole animals. We show that p21^{+/-} and p21^{-/-} mice develop more tumors when irradiated than do p21^{+/+} mice. A greater incidence of Harderian gland and ovarian tumors and a wider spectrum of tumor types contributed to the overall increase in tumor incidence in p21^{+/-} and p21^{-/-} mice, as compared with p21^{+/+} mice. Because p21^{Cip1} was expressed in a large sampling of tumors from p21^{+/-} mice, we suggest that the p21^{Cip1} gene is haploinsufficient for tumor suppression in irradiated mice. Our results are in accordance with previous reports showing enhanced susceptibility of p21^{Cip1}-null mice to chemically induced carcinogenesis (11, 12) and haploinsufficient tumor suppression by p21^{Cip1} in mice expressing a mutant allele of the APC tumor suppressor gene (14) or the v-Ras oncoprotein (13). Our results

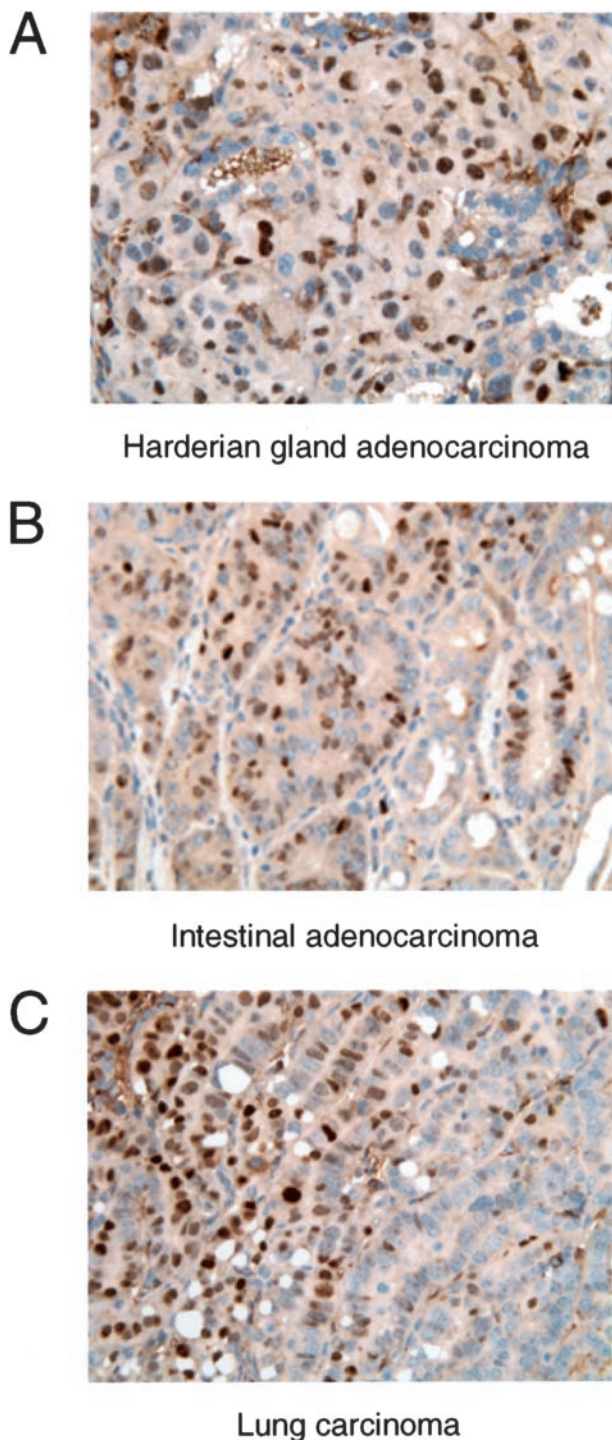


Fig. 1. Immunohistochemical verification of p21^{Cip1} expression in malignant tumors of irradiated p21^{+/-} mice. A, a Harderian gland adenocarcinoma. Magnification, $\times 40$. B, an intestinal adenocarcinoma. Magnification, $\times 40$. C, a bronchioloalveolar carcinoma. Magnification, $\times 40$. Brown staining, presence of p21^{Cip1}.

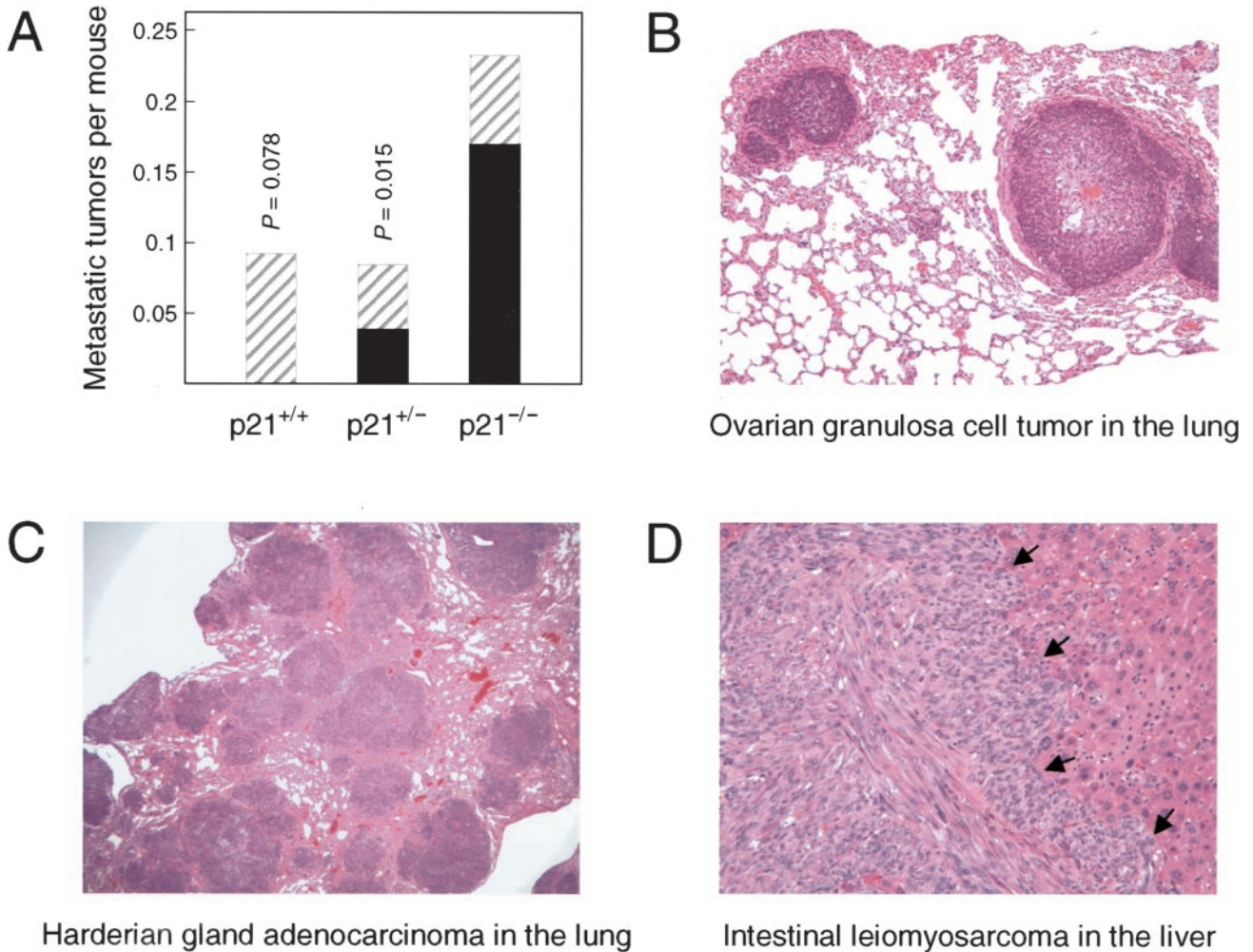


Fig. 2. Metastatic tumors in irradiated mice. *A*, graphic representation of metastatic tumor incidence in irradiated p21^{+/+}, p21^{+/-}, and p21^{-/-} mice. *Solid black bars*, number of metastatic tumors per mouse, excluding thymic lymphomas. *Striped gray bars*, number of thymic lymphomas per mouse. The *Ps* compare p21^{+/+} and p21^{+/-} mice with p21^{-/-} mice and do not include thymic lymphomas. *B-D*, examples of metastatic tumors in p21^{Cip1}-null mice. *B*, an ovarian malignant granulosa cell tumor that metastasized to the lung. Magnification, $\times 2.5$. *C*, extensive pulmonary metastases from a Harderian gland adenocarcinoma. Magnification, $\times 10$. *D*, an intestinal leiomyosarcoma that metastasized to the liver. *Arrows*, tumor margins. Magnification, $\times 20$.

differ, however, from those of Martin-Caballero *et al.* (16), who observed a protective effect of p21^{Cip1} nullizyosity on the survival of γ -irradiated mice. In the study of Martin-Caballero *et al.* (16), 35 mice received a 1.75-Gy dose of radiation once a week for 4 weeks. As a

result, all of the wild-type mice developed T-cell lymphomas and did not survive >10 months. In contrast, in our study, ~ 200 mice received a single 4-Gy dose of radiation, and most mice were lymphoma free and viable at 14 months. Thus, our study allowed us to detect a

Table 2 Metastatic tumors in γ -irradiated p21^{+/+}, p21^{+/-}, and p21^{-/-} mice

Primary organ	Tumor type	Sites of metastasis	Number of metastatic tumors (Tumors per mouse)		
			p21 ^{+/+}	p21 ^{+/-}	p21 ^{-/-}
Adrenal gland	Pheochromocytoma	Liver, lung	0	1 (0.010)	0
Harderian gland	Adenocarcinoma	Lung	0	0	1 (0.016)
Liver	Hepatoblastoma	Intrahepatic	0	0	1 (0.016)
	Histiocytic sarcoma	Pancreas, testes	0	0	1 (0.016)
Lung	Hemangiosarcoma	Brain	0	0	1 (0.016)
Ovary ^a	Granulosa cell tumor	Lung	0	0	1 (0.031)
Small intestine	Adenocarcinoma ^b	Liver, Lung	0	0	3 (0.049)
	Leiomyosarcoma	Liver	0	1 (0.010)	1 (0.016)
Spleen	Hemangiosarcoma	Liver, uterus	0	0	1 (0.016)
Undetermined	Osteogenic sarcoma	Liver, lung	0	1 (0.010)	0
	Leiomyosarcoma	Lymph node	0	1 (0.010)	0
Thymus	Lymphoma	Kidney, liver, spleen	3 (0.097)	5 (0.048)	4 (0.066)
Metastatic tumors per mouse			0.1	0.09	0.24
Metastatic tumors per mouse excluding lymphomas			0	0.04	0.18

^a For metastatic tumors originating in the ovary, the number of tumors per mouse is based on the number of p21^{-/-} female mice.

^b The percentage of intestinal adenocarcinomas that metastasized was significantly greater in p21^{-/-} mice than in p21^{+/-} mice (*P* = 0.041).

wide variety of tumor types in irradiated p21^{+/+}, p21^{+/-}, and p21^{-/-} mice and to accurately assess tumor burden.

The most intriguing finding of our study is the increased tendency of p21^{-/-} tumors to metastasize, as compared with p21^{+/+} and p21^{+/-} tumors. Invasive, malignant tumors give rise to metastatic tumors by a multistep process in which tumor subclones detach from the primary tumor, invade surrounding tissue, disseminate through the bloodstream to distant organs, and grow and vascularize in new locations (19, 20). The inability of irradiated p21^{Cip1}-null cells to arrest in G1 and the consequent replication of damaged DNA may result in the aberrant expression of gene products that induce or inhibit metastasis. Alternatively, loss of p21^{Cip1} and consequent dysregulated cyclin-dependent kinase activity and cell cycle progression may directly facilitate the growth of new tumors at sites of metastasis. Determination of how p21^{Cip1} suppresses metastasis represents an exciting challenge and may facilitate the design of efficacious anti-cancer strategies.

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