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Loss of Nuclear *p21*^{Cip1/WAF1} During Neoplastic Progression to Metastasis in γ -Irradiated *p21* Hemizygous Mice

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Abstract

p21^{Cip1/WAF1} localizes to the nucleus in response to γ -irradiation induced DNA damage and mediates a G₁ checkpoint arrest. Although γ -irradiated *p21*^{+/-} mice develop a broad spectrum of tumors, γ -irradiated *p21*^{-/-} mice develop significantly more metastatic cancers. To evaluate the expression of *p21* in tissues prone or resistant to tumorigenesis as a function of γ -irradiation, and to determine whether phenotypic loss of *p21* heterozygosity occurs during tumor progression in *p21*^{+/-} mice, tissues and tumors from γ -irradiated mice were evaluated immunohistochemically. The percentage of tumors in *p21*^{+/-} mice that were nuclear *p21*-positive declined with progression to metastasis ($p < 0.0001$). Benign tumors were more often *p21*-positive and comprised of larger subsets of nuclear *p21*-positive cells than were malignant tumors of the same histopathological type, while metastatic cancers were nuclear *p21*-negative ($p = 0.0003$). Even when a primary cancer was comprised of a subset of nuclear *p21*-positive cells, the metastatic foci of that same cancer were nuclear *p21*-negative. Mesenchymal tumors, though rare, were more likely metastatic than were epithelial tumors ($p = 0.0004$), and these were invariably nuclear *p21*-negative. Prepubescent epithelial tissues from which most tumors later originated in mice with reduced *p21* gene dosage, (i.e., harderian gland, ovary, small intestine, and lung) were *p21* expressive within 4 hours of γ -irradiation ($p = 0.0625$), so that *p21*/Ki67 ratios increased post- γ -irradiation ($p = 0.03$). In contrast, *p21* did not localize to nuclei of cortical thymocytes, a tissue where tumorigenesis was not augmented by reduced *p21* gene dosage.

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Cellular subclones of malignant tumors, especially those of mesenchymal cell origin, which lack nuclear p21 may more readily acquire the genetic alterations of the metastatic phenotype.

Keywords

p21^{Cip1/WAF1}; *p21* knockout mice; metastasis; immunohistochemistry; tumorigenesis

INTRODUCTION

Neoplastic transformation and progression are phenotypic endpoints of acquired genetic alterations, so processes that activate a cell and move it through each cycle must be precisely controlled to ensure genomic integrity and limit cancer risk. Fidelity is achieved in part by the orderly expression of cyclins that activate cyclin-dependent kinases (CDK). These holoenzymes phosphorylate substrates, which elicit DNA replication and repair, nuclear envelope breakdown, spindle assembly, and chromosome segregation [1-3]. CDKs are constrained by CDK inhibitors (CKI), assigned to either the INK4 family (i.e., *p15^{INK4b}*, *p16^{INK4a}*, *p18^{INK4c}*, and *p19^{INK4d}*), or to the Cip/Kip family (i.e., *p21^{Cip1/WAF1}*, *p27^{Kip1}*, and *p57^{Kip2}*), the later of which inhibit a broad spectrum of cyclin/CDKs.

p21^{Cip1/WAF1} is the principle effector of the DNA damage response p53-mediated G₁ checkpoint arrest, and a contributor to cellular differentiation and senescence [4-8]. Its amino-terminus binds cyclin E- and A-dependent CDK2, while its carboxy-terminus binds proliferating cell nuclear antigen (PCNA), an activator of DNA polymerase- δ , inhibiting DNA replication without modulating DNA repair [8,9]. p21 targets complexed holoenzyme to the nucleus [10], so that its nuclear presence detected immunohistochemically, correlates with its cell cycle inhibitory property [3,11-15].

Cells that incur genetic damage, arrest and repair the damaged DNA, or self ablate, or risk developing neoplastic autonomy. Cell cycle delay in response to γ -irradiation is comprised of a G₁-S transition block, an S phase delay, or a G₂-M arrest, depending on cell type, growth conditions, irradiation dose, and operative checkpoint controls [16,17]. γ -Irradiated cells develop DNA double-strand breaks, detected by Ku heterodimers, and signaled by ataxia telangiectasia mutated (ATM) and ATM-Rad3 related kinase activation of p53 [18]. The p53-transactivation of p21 triggers a cell cycle arrest that permits repair of DNA by homologous recombination [5,6,19,20].

Metastases, not primary tumors, are responsible for most cancer deaths. Although *p21* is rarely mutated in human tumors [21-23], reductions in p21 contribute to neoplastic progression in some tumor sites [24-31]. Hypermethylation of the *p21* promoter in bone marrow cells correlates with low p21 mRNA levels, and serves as a prognostic factor linked to reduced survival of acute lymphoblastic leukemia patients [24]. Reduced and topologically dysregulated p21 protein expression occurs in dysplastic crypts early in colorectal neoplasia [25,26]. Low tumor p21 protein levels, perhaps due to *p21* gene inactivation by histone deacetylation [27,28], is associated with reduced colorectal cancer patient survival [29]. Cytoplasmic translocation of nuclear p21 and diminished restraint of cyclin/CDKs may occur subsequent to *Her-2/neu* gene amplification in breast carcinogenesis [30,31].

As a tumor cell expands to form a clone, descendants begin to diversify so that clinically recognizable cancers are comprised of heterogenous subclones each with its own complement of DNA damage. Cancers metastasize when a particular subclone accumulates genetic alterations that endow it with enhanced growth, invasive, and motility properties, which disrupt the cancer-host interface and permit it to seed and survive at a distant site, while by passing

host defenses [32,33]. Benign tumor cells with demonstrable nuclear p21 may be constrained from progressing to metastasis [24-27]. A larger subpopulation of nuclear p21-positive, cycle-arrested cells is present in non-familial colorectal adenomas compared to carcinomas [34], or lymph node metastases [35]. Lower histological grade breast carcinoma is comprised of a larger subset of nuclear p21-positive tumor cells [36,37]. Whether, as a rule, the subset of cells that express nuclear p21 is larger in primary cancers compared to metastatic foci is not known.

In mouse models, *p21* nullizygosity accelerates the inherent tumorigenesis of the pituitary gland in *Rb*-hemizygous mice [38] and *p18^{INK4c}*-nullizygous mice [39], intestine in *Apc*-hemizygous mice [40], and mammary gland in MMTV-*ras* transgenic mice [41,42]. *p21* nullizygosity increases susceptibility to papilloma or carcinoma [43,44], or anaplastic spindle cell carcinoma [45] in carcinogenesis models of the skin, and increases lung tumor multiplicity subsequent to urethane exposure [46].

We recently reported that *p21* is haploinsufficient for tumor suppression in γ -irradiated mice, and that *p21* nullizygosity increases the incidence of metastatic disease [47]. A broad spectrum of tumor pathologies developed, including significantly more benign epithelial neoplasms of the ovary and harderian gland in γ -irradiated *p21*^{+/+} and *p21*^{-/-} mice. Although p21 expression in primary malignant tumors of *p21*^{+/+} mice was verified immunohistochemically in that prior report, no evaluation of the patterns of p21 nuclear localization as a function of γ -irradiation or tumor progression was made. Herein, we provide evidence of the phenotypic loss of *p21* heterozygosity in tumors of *p21*^{+/+} mice that progress to malignancy and metastasis. These observations suggest that primary cancerous subclones lacking nuclear p21 protein may more likely form metastatic foci.

MATERIALS & METHODS

Animals

Tumors were derived from mice previously described [47], in accordance with the *Guide for Care and Use of Laboratory Animals* (NIH publication 85-23), in a protocol approved by the *Institutional Animal Care and Use Committee*. Mice were *p21* genotyped by polymerase chain reaction [47]. The sequences of primers used and amplicon sizes were for a *p21* common sequence, AAGCCTTGATTCTGATGTGG; for a *p21* wild type sequence, TGACGAAGTCAAAGTTCCAC, generating a 900 bp fragment; and for a *p21* mutant sequence, GCTATCAGGACATAGCGTTG, generating a 750 bp fragment.

Treatment

In our prior study [47], 31 *p21*^{+/+}, 105 *p21*^{+/+}, and 61 *p21*^{-/-}, or 197 total 2-week-old mice were exposed to a 4 Gy dose of γ -irradiation from a ¹³⁷Cs source (J. L. Shephard, Inc., San Fernando, CA.) and euthanatized when tumors developed, or when 56 weeks old. For the present study, twelve additional 2-week-old *p21*^{+/+} mice, two of each sex at each of three intervals, were euthanatized either without being γ -irradiated or at 1 hour or 4 hours after γ -irradiation to assess basal and γ -irradiation induced p21 and Ki-67 staining patterns.

Tumors were identified during comprehensive necropsy. As previously described [47], *p21*^{+/+} mice developed only 1.11 tumors/mouse, while *p21*^{+/+} mice developed 2.23 tumors/mouse, and *p21*^{-/-} mice developed 2.22 tumors/mouse. Further, *p21*^{-/-} mice developed 2.7-fold more metastatic cancers than did *p21*^{+/+} or *p21*^{+/+} mice. Of those tumors that developed, 102 were immunostained, 12 from *p21*^{+/+} mice, 82 from *p21*^{+/+} mice, and 8 from *p21*^{-/-} mice. All tumors and tissues of *p21*^{-/-} mice were negative for p21 staining. Absence of p21 expression in *p21*^{-/-} mice and comparable p21 expressions in *p21*^{+/+} and *p21*^{+/+} mice were confirmed by

Western analysis of embryonic fibroblasts exposed to 4 Gy γ -irradiation (data not shown). Staining patterns were similar for comparable tumor types derived from $p21^{+/+}$ or $p21^{+/-}$ mice.

Immunohistochemistry

Tumors and tissues were sectioned at 3 μ m, deparaffinized, hydrated, and immunostained using a mouse to mouse kit (Chemicon, Temecula, CA.). Endogenous peroxidase was blocked with 3% hydrogen peroxide. Sections were exposed to antibodies for either p21 (1:50, cat. #550827, Pharmingen, San Diego, CA.), Ki-67 (1:100, cat. #556003, Pharmingen), smooth muscle actin (1:100, cat. #AM-0995-11, Innogenex, San Ramon, CA.), CD3 ϵ (1:400, cat. #sc-1127, Santa Cruz Biotechnology), vimentin (1:200, cat. #sc-7557, Santa Cruz Biotechnology), or keratin AE1/AE3 (1:200, cat. #MU071-UC, BioGenetics), or mouse IgG as negative control. Sections were stained with a 1:200 biotinylated anti-IgG secondary antibody (Pharmingen), developed using an avidin-biotin complex kit with diaminobenzidine tetrahydrochloride as chromogen, and counterstained with hematoxylin. Sections were assessed for the % nuclei that immunostained for p21 or Ki-67 by counting 1000 nuclei in representative fields. Immunostaining was judged negative if <3%, rare if 3-5%, minimal if 6-25%, moderate if 26-50%, and marked if >50% of the nuclei stained. Nuclear presence of p21 correlates with its cell cycle inhibitory property. Alternatively, Ki-67 is localized in nuclei of actively cycling cells. Since the proportion of cells that stain for either p21 or Ki-67 varies greatly among tumor types, comparisons were made of p21/Ki-67 ratios, that is the proportion of tumor cells that were cycle arrested/pool of actively cycling cells. No appreciable level of staining was evident with IgG negative controls.

Statistical Analysis

Constitutive and γ -irradiation induced p21 or Ki67 immunostaining, and ratios of % p21-positive to % Ki67-positive cells were compared using Wilcoxon signed ranks test. Numbers of tumors per mouse, not previously reported [47], were compared in two-way and three-way Kruskal-Wallis tests, and one-way ANOVA. Statistical comparisons of proportions of tumor types were based on Fischer's exact test. All reported p-values are two-sided.

RESULTS

A broad spectrum of 82 tumors from $p21^{+/-}$ mice were analyzed for p21 and Ki67 expressions. In our prior study, tumor incidence, multiplicity, and spectrum were greater in $p21^{+/-}$ and $p21^{-/-}$ mice compared to controls [47]. Only $p21^{+/-}$ and $p21^{-/-}$ mice developed a broad spectrum of mesenchymal tumors other than lymphoma ($p=0.066$, three-way analysis). In contrast to the principally benign epithelial tumors that developed, most mesenchymal tumors were malignant neoplasms (67%). Further, only 8% of epithelial cancers compared to 86% of mesenchymal cancers were metastatic ($p<0.0001$) (Figure 1). Mesenchymal cancers comprised only 4% of the total tumors, but 73% of the metastatic cancers that developed. Even when malignant lymphoma, which were invariably disseminated, were excluded from analysis, nonlymphoid mesenchymal cancers were still more likely to metastasize than epithelial cancers ($p=0.0004$). Since only $p21^{-/-}$ mice developed a broad spectrum and significantly more metastatic cancers compared to $p21^{+/+}$ and $p21^{+/-}$ mice [47], those metastatic cancers that developed in $p21^{+/-}$ mice were immunostained for nuclear p21 to determine whether phenotypic loss of p21 heterozygosity accompanies tumor progression to metastasis.

Tumor Immunohistochemistry

Of the 82 primary tumors from $p21^{+/-}$ mice analyzed for p21 and Ki67 expression, 40 were benign and 42 were malignant tumors (Table I). Nine of the malignant tumors were metastatic cancers, of which five were lymphomas. The percent of tumors from $p21^{+/-}$ mice that were positive for nuclear p21 declined with progression to metastasis ($p<0.0001$, three-way

comparison). Although 83% of benign tumors were comprised of at least 3% of cells that were nuclear p21-positive, only 62% of malignant tumors were (p=0.1, two-way comparison), while none of the metastatic cancers were nuclear p21-positive (p=0.0003, two-way comparison).

Tumors did not stain uniformly. Some tumor types had clusters of cells with moderate to marked p21 staining along advancing margins (e.g., bronchioloalveolar carcinoma) or near luminal surfaces (e.g., small intestinal adenocarcinoma) (Figure 2). Other tumor types had p21-positive cells spread throughout the tumor at moderate (e.g., harderian gland adenocarcinoma) or minimal levels (e.g., malignant granulosa cell tumor). Cells of the adjacent normal lining epithelium or normal parenchyma of adult mice rarely stained for nuclear p21.

The percent of cells that stained in each tumor type were highly variable. In general, benign tumors were comprised of larger subsets of nuclear p21-positive cells and smaller subsets of Ki67-positive cells. Expressed as a ratio of p21-positive to Ki67-positive cells, benign tumors had somewhat larger ratios than malignant tumors of the same histopathological type, and tissue-specific trends were evident (Table I). For example, harderian gland adenoma and carcinoma were each comprised of comparably sized subsets of cells that stained for either nuclear p21 or Ki67, so that p21/Ki67 ratios were similar, or 0.98 and 0.81, respectively (Figure 3). Other tumor types tended to have minimal p21, but marked Ki67 immunostaining. For example, small intestinal adenoma and adenocarcinoma were characterized by a moderate p21-positive pool of cells clustered near the luminal surface, but a much larger Ki67-positive pool of cells throughout the neoplasm, so that the p21/Ki67 ratios were 0.12 and 0.10, respectively.

Of the nine metastatic or disseminated cancers that developed in $p21^{+/-}$ mice, five were CD3ε-positive malignant lymphoma, which were markedly Ki67-positive, and invariably negative for nuclear p21 (Figure 4), as were those which developed in three $p21^{+/+}$ mice. Four other metastatic cancers developed in four separate $p21^{+/-}$ mice, one pheochromocytoma, one osteogenic sarcoma, and two leiomyosarcomas.

Both of the leiomyosarcoma were negative for p21, but comprised of a moderate replicating pool of Ki67-positive tumor cells. In a widely disseminated osteogenic sarcoma of undetermined primary site, all 19 pulmonary metastatic foci and 14 of 15 hepatic metastatic foci lacked p21-positive cells, while a single hepatic metastatic focus had rare, faintly p21-positive cells. Osteogenic sarcoma cells stained moderately for Ki-67.

In a metastatic adrenal pheochromocytoma, nuclear p21 was localized in 11% of the primary tumor cells, with a moderate 26% of p21-positive cells clustered along the advancing edge (Figure 5). Only 6% of the primary tumor cells stained for Ki67. Cancer cells of the two hepatic metastatic foci, and 8 of 10 pulmonary metastatic foci were negative for nuclear p21, while the other 2 pulmonary metastatic foci had rare, faintly p21-positive cells and 3-25% of cells of these metastases stained for Ki67.

Tissue Immunohistochemistry

Since $p21^{+/-}$ and $p21^{-/-}$ mice developed more epithelial tumors, but a comparable number of malignant lymphoma compared to $p21^{+/+}$ mice, we analyzed the p21 and Ki67 staining patterns of these and other tissues as a function of 4 Gy γ -irradiation.

Prior to γ -irradiation, nuclear p21 was detected in only minimal cells of the epididymis, oviduct, and exocrine pancreas, and in rare epithelial lining cells of the intestinal upper crypts and lower villi, urinary bladder, and the supra-basal zone of the skin, esophagus, and aglandular stomach. Ki67 nuclear staining was evident in a moderate 26-50% of replicating parenchymal and epithelial lining cells of many organs from the prepubescent mice, and a marked >50% of thymocytes, splenocytes, and nodal lymphocytes.

Four hours after γ -irradiation, nuclear p21 was localized to significantly more cells compared to pre-exposure levels in the adrenal gland, epididymis, aglandular and glandular stomach, harderian gland, large intestine, lung, ovary, oviduct, pancreas, small intestine, skin, urinary bladder, uterus, and vagina (each tissue, $p=0.0625$) (Figure 6). Scattered hepatocytes, myocytes, cardiomyocytes, endothelial cells, and stromal fibroblasts stained for nuclear p21 after γ -irradiation.

In contrast, nuclear p21 was not localized to cells of the cortical thymus, white pulp of the spleen, lymph node cortex, or Peyer's patch lymph nodules before or after γ -irradiation. Apoptosis was a prominent histological feature after γ -irradiation in these tissues. Four hours after γ -irradiation, only rare (3-5%), scattered, heterogeneous cells of the thymic medulla, splenic red pulp, and lymph node medulla, including reticular epithelial cells, endothelial cells, and some lymphocytes, stained for nuclear p21. Although apoptotic cells and bodies were occasionally noted in focal areas of other tissues, including the deep crypt epithelium of the intestine, apoptosis was a prominent feature of only the thymus, spleen, and lymph node after γ -irradiation.

Following γ -irradiation, tissues prone to tumorigenesis with reduced *p21* gene dosage (i.e., harderian gland, ovary, small intestine and lung) showed a modest reduction in the Ki67-positive pool. The ratios of % p21-positive to % Ki67-positive cells of each of these four tissues increased following γ -irradiation ($p=0.03$). In contrast, in the cortical thymus, where reduced *p21* gene dosage modestly lowered incidence and delayed recognition of malignant lymphoma [47], thymocytes did not stain for p21 while the large Ki67-positive proliferative pool remained constant.

DISCUSSION

Whether the metastatic potential of a tumor is established early and encoded in a majority of cells before the primary tumor becomes clinically recognized [48], or develops late in a rare malignant subclone that acquires the capacity to metastasize [49] is not clear. Perhaps a metastatic phenotype accumulates more readily in tumor cells that prematurely transition from G₁ to S phase without an effective opportunity for DNA repair. Theoretically, when mice are γ -irradiated, cells in tissues that are nuclear p21 responsive arrest in G₁ to repair DNA damage [4-8,20]. With inactivation of *p21*, the cyclin D-CDK4 link between mitogenic growth factor cues and the autonomous cell cycle with activated cyclin E-CDK2 operates under-restrained, increasing the likelihood that cells will inherit genetic aberrations of the antecedent cell. Our previous [47] and present findings support the premise that *p21* nullizygosity or loss of nuclear p21 in tumor cells of *p21*^{+/-} mice potentiates tumor progression to metastasis. Alterations of cell cycle regulatory genes may contribute to a mutator phenotype that acquires genetic lesions, evolving toward a metastatic phenotype.

Whether aspects of the metastatic phenotype accumulate preferentially in cells lacking nuclear p21 is not known. Loss of the *p53*-dependent cyclin-CDK-PCNA inhibitory aspects of p21 in renewable cell populations may contribute to tumor progression by preventing normal cell differentiation and senescence. p21 contributes to the terminal differentiation of certain cell types including myocytes, oligodendrocytes, skin and intestinal epithelia, and hematopoietic cells in a manner independent of *p53* [50-53]. In the present study, myocytes in γ -irradiated prepubescent mice showed upregulation of nuclear p21. Mice developed leiomyosarcoma only in *p21*^{+/-} or *p21*^{-/-} cohorts, three of which were widely disseminated, two of which were anaplastic spindle cell tumors with some differentiation. None had a subset of nuclear p21-positive cells. Loss of nuclear p21 may prevent normal mesenchymal cell differentiation and senescence, diminished G₁ checkpoint control, and opportunity for tumor progression.

Epithelial tissues where benign tumorigenesis was significantly increased by reduced p21 gene dosage had nuclear p21/Ki67 cell ratios that increased in response to γ -irradiation. In contrast, nuclear p21 did not localize to cortical thymocytes. Recognition of malignant lymphoma was delayed and reduced somewhat in $p21^{+/-}$ mice (incidence 0.048; mean age 49 ± 14 weeks), and $p21^{-/-}$ mice (incidence 0.066; mean age 38 ± 17 weeks), compared to $p21^{+/+}$ mice (incidence 0.097; mean age 28 ± 9 weeks) [47]. Loss of $p21$ delays the onset of malignant lymphoma following γ -irradiation [54], or in an ATM-deficient context [55]. Thymocytes undergo massive apoptosis, and express high levels of p21 mRNA in a $p53$ -dependent manner in response to γ -irradiation, although p21 may not be required for thymocyte apoptosis [56, 57].

Translocation of nuclear p21 to the cytoplasm is accompanied by an alteration of its physiologic function, perhaps promoting cell survival by resisting apoptosis. Full-length nuclear p21 translocates to the cytoplasm following differentiation of U937 cells into monocytes, where it complexes with apoptosis signal-regulating kinase 1, inhibiting the stress-induced mitogen-activated protein kinase cascade, and eliciting a resistance to apoptotic stimuli [58]. *HER-2/neu* over-expression in fibroblasts activates the phosphatidylinositol-3 kinase/Akt pathway, resulting in the phosphorylation and cytoplasmic translocation of p21, and the suppression of its cell cycle inhibiting activity [30]. Alternatively, processed and translocated p21 may augment apoptosis. In growth factor-deprived endothelial cells, cleavage by caspase-3 results in a truncated p21 lacking a nuclear localization signal, causing it to locate to the cytoplasm, releasing cyclin A-CDK2 from constraint, and augmenting apoptosis [59].

Further studies should evaluate whether thymocytes are nuclear p21 responsive, whether thymic p21 highly induced by γ -irradiation is processed, and/or translocated to the cytoplasm, where it may increase the resistance of cancerous subclones to apoptosis, and precipitate the recognition of lymphoma in $p21^{+/+}$ mice.

Studies are also needed to determine the mechanisms of tumor cell progression to which loss of nuclear p21 contributes. Recently, absence of p21 has been shown to cooperate with *c-myc* in bypassing *ras*-induced senescence, and increase oncogenic cooperation between *ras* and *myc* [60]. Additional studies should determine whether the phenotypic loss of p21 heterozygosity during tumor progression in γ -irradiated mice is attributable to alteration of the remaining $p21$ allele, or a silencing of $p21$ due to promoter hypermethylation.

Although no judgement can be made about the probability of metastasis from the pathological examination of the primary tumor, and no single genetic alteration renders a cancer cell metastasis-prone, alterations to the cell cycle contribute to accumulation of genetic aberrations that drive neoplastic transformation and progression [61]. Diminished p21-mediated G_1 checkpoint control and cell differentiation may contribute to acquisition of the molecular basis of the metastatic phenotype.

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Figure 1.

A broad spectrum of metastatic cancers developed in γ -irradiated mice only in the absence of p21 expression, either in $p21^{-/-}$ mice or p21-negative cancer metastases of $p21^{+/-}$ mice. Most metastatic cancers were mesenchymal in origin. Epithelial cancers that were metastatic included an (A) adrenal pheochromocytoma metastatic to the lung, a (B) Harderian gland adenocarcinoma metastatic to the lung, a (C) hepatoblastoma with multiple intra-hepatic metastases, a (D) mucicarmine-negative, faintly keratin-positive undifferentiated carcinoma metastatic to the liver from a mouse with a small intestinal adenocarcinoma as the only detectable primary cancer, and a (E) malignant granulosa cell tumor metastatic to the lung. More metastatic mesenchymal cancers developed in γ -irradiated $p21^{+/-}$ and $p21^{-/-}$ mice, including a (F) leiomyosarcoma of undetermined primary site metastatic to the lymph node, an (G) osteogenic sarcoma of undetermined primary site metastatic to the liver, several (H) thymic malignant lymphoma here infiltrating the lung, a (I) hemangiosarcoma metastatic to the lung, a (J) histiocytic sarcoma metastatic to the pancreas, and an (K) anaplastic spindle cell tumor that effaced normal hepatic parenchyma, surrounded a remnant cord of hepatocytes (arrows), and exhibited smooth muscle differentiation (upper left), and in other areas of the same cancer appeared as a well-differentiated (L) leiomyosarcoma metastatic to the liver. Mouse genotype is indicated above each tumor.

Figure 2. Nuclear p21 expression levels and patterns in individual primary tumors. Clusters of p21-positive cells were present along advancing margins of (A) bronchioloalveolar carcinoma, and near the luminal surface of (B) small intestinal adenocarcinoma, or were present diffusely throughout a (C) harderian gland adenocarcinoma at moderate levels, or a (D) malignant granulosa cell tumor at minimal levels.

Figure 3.

Subjacent sections of primary tumors immunostained for p21 or Ki-67. A comparable subset of tumor cells of a harderian gland adenoma immunostained for (A) nuclear p21 or (B) Ki-67, but cells of the adjacent normal glandular parenchyma (left edge of each A & B) rarely stained for either marker. In contrast, (C) a clustered subset of tumor cells near the luminal surface (arrow) of a small intestinal adenocarcinoma immunostained for nuclear p21, but most tumor cells did not, and only rare cells of the adjacent normal mucosa did (rectangle in upper right), while (D) most tumor cells and numerous cells of the adjacent normal mucosal crypts and lower villi stained for Ki-67.

Figure 4.

Subjacent sections of metastatic cancers immunostained for p21 (A, C, E) or Ki-67 (B, D, F). Malignant lymphoma (A), here shown infiltrating the kidney, were negative for nuclear p21, although an occasional renal tubular cell nucleus (arrow) was nuclear p21-positive, and (B) most malignant lymphoma cancer cells were Ki67-positive. Cancer cells of all nineteen pulmonary metastatic foci of a metastatic osteogenic sarcoma (C) were negative for nuclear p21, although (D) a moderate subpopulation of osteogenic sarcoma cancer cells were Ki67-positive. Fewer than 3% of cancer cells of a lymph node metastatic focus of a leiomyosarcoma (E) stained faintly for nuclear p21, but these same cancer cells (F) were moderately Ki67-positive.

Figure 5.

Nuclear p21 and Ki67 immunostaining levels and patterns of an adrenal gland pheochromocytoma (A-C) that was metastatic to the lung (D-F) and liver (G-I). Metastatic pheochromocytoma cancer cells (arrows) compressed the adjacent normal pulmonary (D) and hepatic (G) parenchyma. Clusters of primary tumor cells were moderately p21-positive (B) and Ki67-positive (C). In contrast, metastatic cancer cells were negative for nuclear p21 (E & H), but moderately Ki67-positive in pulmonary metastatic foci (F), or rarely Ki67-positive (I, arrows) in hepatic metastatic foci.

Figure 6.

Nuclear p21 prior to (A, D, G, J) and 4 hours after (B, E, H, K) γ -irradiation, and Ki67 (C, F, I, L) expression 4 hours after γ -irradiation in prepubescent mice. Prior to γ -irradiation, nuclear p21 was only localized in limited tissues, including minimal cells of the oviduct (D, arrow) or exocrine pancreas (G, lower arrow), or in rare cells of the small intestinal crypts (G, upper arrow), but was not present in the cortical thymus (J, left of diagonal). Epithelial tissues prone to tumorigenesis with reduced *p21* gene dosage were comprised of a moderate subset of nuclear p21-positive cells after γ -irradiation (B, E, H), but the cortical thymus was not (K, left of diagonal). Nuclear p21 was localized in (B) epithelial cells of the harderian gland ducts and alveoli, in (E) follicular granulosa and theca cells of the ovary, and in (H) mucosal epithelial

cells of the upper crypt and lower villus of the small intestine, as well as acinar cells of the exocrine pancreas (H, lower left), and in rare, scattered, heterogeneous cells of the thymic medulla (K, right of diagonal), but not in cortical thymocytes (K, left of diagonal) after γ -irradiation, where numerous apoptotic cells and bodies were evident. Each tissue had moderate to marked pools of replicating Ki67-positive cells (C, F, I, L).

Table I

Nuclear p21 Localization in Tumors of *p21*^{+/-} Mice

Primary Organ	Classification of Tumors		# p21-positive/ # evaluated (ratio) ²	Immunostaining of Tumor Cells		
	Morphological Diagnosis ¹			% p21-positive ³	% Ki67-positive ³	Ratio ⁴
Adrenal gland	Phaeochromocytoma*		1/1 (1.0)	11.2	6.1	1.8
Harderian gland	Adenoma		8/8 (1.0)	26.6 ± 13.0	27.2 ± 12.5	0.98
Liver	Adenocarcinoma		4/5 (0.8)	25.4 ± 19.1	31.4 ± 10.5	0.81
	Hepatocellular carcinoma		0/1	<3	8.1	0
Lung	Bronchioalveolar adenoma		2/3 (0.67)	4.9 ± 1.4	6.7 ± 1.6	0.73
	Bronchioalveolar carcinoma		4/7 (0.57)	3.8 ± 1.1	8.8 ± 3.2	0.43
Ovary	Adenoma, tubulostromal		0/3	<3	6.7 ± 3.2	0
	Adenoma, gonadal stromal		6/7 (0.86)	11.5 ± 6.7	25.0 ± 12.7	0.46
Pituitary gland	Granulosa cell tumor, malignant		1/3 (0.33)	3.6	22.0 ± 10.8	0.16
	Adenoma		1/1 (1.0)	6.3	11.3	0.56
Salivary gland	Hemangiosarcoma		0/1	<3	13.8	0
	Adenoma		16/18 (0.89)	7.5 ± 6.9	62.5 ± 10.1	0.12
Small Intestine	Adenocarcinoma		16/16 (1.0)	7.1 ± 5.8	71.0 ± 8.4	0.10
	Lefomyosarcoma*		0/1	<3	36.3	0
Thymus	Lymphoma, malignant*		0/5	<3	96.2 ± 3.7	0
	Osteogenic sarcoma*		37/ 0/1	<3	37.4	0
Undetermined	Lefomyosarcoma*		0/1	<3	39.6	0
Benign Tumors			33/40 (0.83)	9.5	23.2	0.41
Malignant Tumors			26/42 (0.62)	5.1	35.5	0.14
Metastatic Cancers			0/9	<3	47.1	0

¹ Benign tumors (unshaded) were localized, well-demarcated, expansile masses of well-differentiated cells. Malignant tumors (shaded) were invasive masses of pleomorphic cells, some of which were cancers with metastasis (*).

² Number of tumors comprised of ≥3% cells with nuclear p21 per number of tumors evaluated immunohistochemically (ratio).

³ Mean ± SD % of tumor cells that immunostained for nuclear p21 or Ki67.

⁴ Ratio of means; mean % nuclear p21-positive tumor cells per mean % Ki67-positive tumor cells.