[CANCER RESEARCH 58, 114-122, January 1, 1998]

Localization and Expression of $p27^{KIP1}$ in Multistage Colorectal Carcinogenesis[1]

Marco Ciaparrone, Hirofumi Yamamoto, Yao Yao, Alessandro Sgambato, Giorgio Cattoretti, Naohiro Tomita, Takushi Monden, Heidrun Rotterdam, and I. Bernard Weinstein[2]

Centro di Ricerche Oncologicalhe "Giovanni XXXIII," Università Cattolica del Sacro Cuore, 00168 Rome, Italy [M. C., A. S.]; Herbert Irving Comprehensive Cancer Center [I. B. W., H. Y., Y. Y.] and Department of Pathology [G. C., H. R.], Columbia University, College of Physicians and Surgeons, New York, New York 10032; and Department of Surgery II, Osaka University Medical School, 565 Osaka, Japan [T. M., N. T.]

ABSTRACT

The cyclin-dependent kinase inhibitor $p27^{Kip1}$ can inhibit the G_1 to S transition of the cell cycle and is a putative tumor suppressor. However, our laboratory found that a variety of human cancer cell lines express relatively high levels of this protein and that this is often associated with increased expression of cyclin D1 or cyclin E. Therefore, in the present study we analyzed by immunohistochemistry the expression of $p27^{Kip1}$ in a series of human tissue samples representing various stages of colon carcinogenesis, using 20 samples of normal colon mucosa, 20 hyperplastic polyps, 19 samples of adenomatous polyps, and 40 samples of various types of colorectal carcinomas. Parallel immunostaining was done for cyclin D1 and also for Ki67 to evaluate cell proliferation. An additional 17 human colon carcinoma samples, together with paired adjacent normal mucosa samples, were analyzed for levels of expression of the $p27^{Kip1}$ protein by Western blot analysis, and 7 of these pairs of samples were examined by Northern blot analysis for levels of $p27^{Kip1}$ mRNA. We did not find a positive or negative correlation between $p27^{Kip1}$ expression and cell proliferation in the normal mucosa and tumor samples. There was, however, an inverse correlation between $p27^{Kip1}$ and Ki67 expression in the lymphoid follicles present in the colonic mucosa. There was no evidence for a consistent increase or decrease in $p27^{Kip1}$ expression in the mucosal cells during colon carcinogenesis, because the mean values for percentage $p27^{Kip1}$-positive cells were similar in the normal mucosa, adenomatous polyps, and carcinoma samples. This is in contrast to Ki67 and cyclin D1 expression, which did show significant increases in mean values with tumor development. A subset (35%) of the carcinomas displayed diffuse cytoplasmic staining, in addition to nuclear staining, for $p27^{Kip1}$, and in these cases the percentage of cells that were positive for $p27^{Kip1}$ was higher than in cases that had only nuclear staining. There was a significant correlation between $p27^{Kip1}$ expression and tumor grade; *i.e.*, well and moderately differentiated carcinomas had high $p27^{Kip1}$ expression, whereas poorly differentiated carcinomas had lower expression. The Western blot analysis data on $p27^{Kip1}$ expression confirmed this correlation. Comparisons of Northern and Western blots did not show a correlation between the level of $p27^{Kip1}$ mRNA and the corresponding protein, a finding consistent with evidence that the $p27^{Kip1}$ protein is regulated mainly via a posttranscriptional mechanism. The immunostaining studies revealed a significant correlation between high $p27^{Kip1}$ protein expression and high cyclin D1 expression in the adenomatous polyps and in the subset of carcinomas that had only nuclear $p27^{Kip1}$ expression. This may reflect the existence of a homeostatic feedback mechanism that is lost in the high-grade carcinomas that express low levels of $p27^{Kip1}$.

INTRODUCTION

Abnormalities in various components of the cell cycle-regulatory machinery have been found in several types of human cancer. Thus, amplification and/or increased expression of the *cyclin D1* gene have been found in esophageal, head and neck, hepatic, breast, and colon cancers; *cyclin E* is often deregulated in colon, breast, and prostate tumors; and *Cdk*[3] *4* is often overexpressed in sarcomas, gliomas, and colon carcinomas (1–11). There is also increasing evidence for alterations in the expression of specific CKIs in human cancers. Six CKIs, which form two distinct classes, have been identified in mammalian cells. The first class includes the INK4 proteins: $p16^{INK4A}$ (12), $p15^{INK4B}$ (13), $p18^{INK4C}$ (14), and $p19^{INK4D}$ (15). Each of these proteins can form complexes with Cdk4 and Cdk6 (16). Overexpression of INK4 proteins can block cells in G_1 (17), presumably through inhibition of cyclin D/Cdk activity. Inactivation of the $p15^{INK4B}$ or $p16^{INK4A}$ proteins results in accelerated entry into S phase and appears to contribute to cellular transformation, suggesting that the INK4 proteins function as tumor suppressor genes (18). Indeed, a high frequency of $p15^{INK4B}$ and $p16^{INK4A}$ gene deletions occur in specific tumors and in tumor-derived cell lines (15, 19, 20). Moreover inactivation of $p16^{INK4A}$ expression due to hypermethylation of this gene has also been seen in human tumors (21), and $p16^{INK4A}$-deficient mice develop spontaneous tumors at an early stage and are highly sensitive to carcinogens (22).

The second class of CKIs includes $p21^{Cip1}$ (23, 24) $p27^{Kip1}$ (25, 26), and $p57^{Kip2}$ (27). The $p21^{Cip1}$ gene is located on chromosome 6p, and the encoded protein inhibits a variety of cyclin-Cdk complexes, including cyclin D-Cdk4, cyclin E-Cdk2 and cyclin A-Cdk2. Its expression is regulated by the tumor suppressor p53 (28) although it can also be induced by p53-independent mechanisms (29) and may be involved in cellular senescence (30). No mutations in the $p21^{Kip1}$ gene have been described thus far in human cancers (31), and p21-deficient mice undergo normal development, although fibroblasts from these mice are defective in G_1 arrest in response to DNA damage and nucleotide pool depletion (32, 33). Two additional CKIs that are structurally related to $p21^{Cip1}$ have been identified, $p27^{Kip1}$ and $p57^{Kip2}$. The latter gene (27) has not been studied in detail in human cancers. The $p27^{Kip1}$ protein associates with cyclin D-Cdk4, cyclin E-Cdk2, and cyclin A-Cdk2 complexes and can inhibit their activities (34). Several studies have demonstrated the importance of this protein in controlling G_1 progression during the cell-cycle. Thus, $p27^{Kip1}$ interfered with G_1 progression when its level was increased by cAMP agonists in macrophages (35), by rapamycin in T lymphocytes (36), by IFN-γ in mammary epithelial cell lines (37), or by IFN-β in the TMK-1 human gastric cell line (38). On the other hand, abrogation of $p27^{Kip1}$ function suppresses quiescence in Chinese hamster cell lines (39). This protein appears to play a role in both cell growth and differentiation, because ectopic overexpression of $p27^{Kip1}$ induces differentiation in some cell lines (40). It is of interest that the expression of $p27^{Kip1}$ is regulated mainly at the posttranscriptional level via a ubiquitin-proteasome mediated proteolysis mechanism (41, 42). The $p27^{Kip1}$ gene is located on chromosome 12p (43–45). Although the role of $p27^{Kip1}$ in negative regulation of cell proliferation suggests that it may function as a tumor suppressor gene, several authors have noted the absence of mutations in this gene in a variety of tumors

Received 7/25/97; accepted 10/24/97.

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

[1] This research was supported by National Cancer Institute Grant CA 63467 and by an award from the National Foundation for Cancer Research (to I. B. W.). M. C. was supported by an award for biomedical research from the Toniolo Institute, Università Cattolica del Sacro Cuore (Milan, Italy), and H. Y. was supported by an award from FUSO Pharmaceutical Co. (Osaka, Japan).

[2] To whom requests for reprints should be addressed, at Herbert Irving Comprehensive Cancer Center, Columbia University, 701 West 168th Street, New York, NY 10032. Phone:(212) 305-6921; Fax: (212) 305-6889; E-mail: weinstein@cuccfa.ccc.columbia.edu.

[3] The abbreviations used are: Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor.

(43–45). Therefore, this protein may play a positive role in the growth of some tumors.

Indeed, we found that a variety of human carcinoma cell lines express relatively high levels of $p27^{Kip1}$ (46–50). In a series of esophageal cancer cell lines, there was a positive correlation between the level of the cyclin D1 protein, which would be expected to enhance growth, and the level of the $p27^{Kip1}$ protein, which would be expected to inhibit growth (46). Human colon and breast cancer cell lines also expressed high levels of the $p27^{Kip1}$ protein, but this protein was expressed at low levels in three normal mammary epithelial cell lines (47–50). Ectopic overexpression of cyclin D1, or cyclin E, in mammary epithelial cell lines that express low levels of both of these cyclins was associated with increased expression of $p27^{Kip1}$ (47–50). The reciprocal effect was also seen, because when we used an antisense cyclin D1 cDNA to reduce the expression of cyclin D1 in an esophageal or colon cancer cell line, this led to reduced levels of expression of the $p27^{Kip1}$ protein (51, 52). On the basis of these findings, we postulated the existence in some cell types of a feedback inhibitory loop between cyclin D1 or E and $p27^{Kip1}$, the function of which might be to maintain a homeostatic balance between positive and negative regulators of the G_1 to S progression of the cell cycle (46–50, 52).

In view of the above findings, the present study used immunohistochemistry to compare the topology and frequency of expression of the $p27^{Kip1}$ protein to that of cyclin D1 and Ki67, a marker of proliferation (53), in a series of samples of normal human colonic mucosa, hyperplastic polyps, adenomatous polyps, and colorectal carcinomas. Western and Northern blot analyses for levels of expression of the $p27^{Kip1}$ protein and mRNA, respectively, were also done on a smaller set of paired colorectal carcinoma and normal mucosa samples. We also evaluated possible correlations between the findings obtained with the carcinomas and various clinical and pathological parameters. Our findings suggest that $p27^{Kip1}$ plays a complex role in colon carcinogenesis, which is not simply related to its inhibitory effects on cell proliferation.

MATERIALS AND METHODS

Patients. A total of 98 cases were investigated. Patients underwent surgery at the Columbia-Presbyterian Medical Center (New York, NY; 88 cases) or at Osaka University Medical School (Osaka, Japan; 10 cases) from February 1995 to May 1996. The mean age was 67.74 (range, 32–92 years). The tissues were removed endoscopically or obtained at the time of surgery and then routinely processed. They were formalin fixed and paraffin embedded for the immunohistochemical study or immediately frozen at −80°C for the Western and Northern blot analyses. Samples were classified for histological type and Dukes' stage according to standard criteria. Adenomas and colorectal carcinomas were graded according to the WHO grading system. The samples included normal mucosa (n = 35) from the resection margin of colon carcinomas (more than 10 cm away from the tumor), normal mucosa (n = 2) from patients without neoplastic diseases, hyperplastic polyps (n = 20), adenomatous polyps (n = 19), and colorectal carcinomas (n = 57). We selected 20 normal mucosa samples, 20 hyperplastic polyps, 19 adenomatous polyps, and 40 colorectal carcinomas for immunohistochemical analysis. Seventeen pairs of colorectal carcinomas together with adjacent normal colonic mucosa were used for Western blot analysis, and 7 of these pairs of samples were used for Northern blot analysis of $p27^{Kip1}$ expression.

Immunohistochemistry. Representative blocks of formalin-fixed, paraffin-embedded tumor tissue were cut at 4-μm thickness, and then a standard avidin/biotin peroxidase technique was used. In brief, the sections were dewaxed in xylene and then rinsed in alcohol and graded alcohol/water mixtures. Then, 3% hydrogen peroxide was applied to block endogenous peroxidase activity. The sections were subsequently treated in a microwave oven twice for 5 min in citrate buffer (pH 6.0) at high power (750 W). After blocking with 10% goat serum, the primary antibody was applied as follows: the polyclonal antibody to $p27^{Kip1}$ (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a concentration of 2 μg/ml, and the incubation time was 30 min at room temperature; the polyclonal antibody to cyclin D1 (Upstate Biotechnology, Lake Placid, NY) was used at a concentration of 10 μg/ml, and the incubation time was 2 h at room temperature; and the polyclonal antibody to Ki67 (DAKO, Carpinteria, CA) was used at a concentration of 5 μg/ml, and the incubation time was 1 h at room temperature. After rinsing, the biotinylated secondary antibody and horseradish peroxidase-conjugated antibiotin antibody (Vectastain ABC-peroxidase kit, Vector Laboratories, Burlingame, CA) were applied to the sections according to the manufacturer's instructions. Peroxidase activity was visualized by applying the diaminobenzidine chromogen, containing 0.05% hydrogen peroxide, for 3 min. The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted. The above-described precise reaction times permitted consistent reproducibility, thus allowing accurate comparison of all of the samples.

Specificity of Immunostaining. Specificity of $p27^{Kip1}$ staining was assessed first by a preabsorbtion test with the protein used to generate it. This test abolished $p27^{Kip1}$ staining. Second, positive staining of the MCF-7 breast cancer cell line, which has a relatively high level of $p27^{Kip1}$, was used as a positive control. As an internal positive control for preservation of the $p27^{Kip1}$ antigenicity in tissues, we used the strong positive immunostaining of lymphocytes present in the sections examined. The pattern of staining seen with the $p27^{Kip1}$ antibody from Santa Cruz Biotechnology was confirmed on duplicate slides using a monoclonal antibody from Transduction Laboratories (Transduction Laboratories, Lexington, KY). The cyclin D1 primary antibody was a polyclonal IgG-rabbit antibody that reacts with cyclin D1 and D2 but not cyclin D3. A human breast cancer specimen that was known to be positive for cyclin D1 was used as a positive control. For Ki67, immunostaining tonsil samples were used as positive controls. For negative controls, PBS alone was used as substitute for the primary antibody to verify the possibility of false-positive responses from the secondary antibody.

Immunohistochemical Assessment. All of the immunostaining was evaluated in a coded manner without knowledge of the clinical and pathological parameters. For each section, 10 high-power fields were chosen, and a total of at least 1000 cells were evaluated. All slides were interpreted by one of the investigators (M. C.). The results were expressed as the percentage of cells counted that gave positive staining. To confirm reproducibility, 25% of the slides were chosen randomly and scored twice. All duplicates were evaluated similarly. Because the preabsorbtion test eliminated both nuclear and cytoplasmic stainings, cells were scored as $p27^{Kip1}$ positive regardless of the cellular compartment in which the protein was detected. Only cells that were definitely stained were considered positive. To ascertain the level of expression of the $p27^{Kip1}$ protein in normal colonic mucosa, we examined 2 colonic mucosa samples from normal colon samples and 18 samples of histologically normal mucosa from the resected margin of colorectal carcinomas. The $p27^{Kip1}$ staining in these samples showed a wide range of expression, from 5 to 80% with a median value of 50%. Therefore, in the pathological samples, $p27^{Kip1}$ immunoreactivity was considered low if the percentage of positive cells was below the median value of 50%, and it was considered high if the percentage of positive cells was equal to or greater than 50%. This cutoff value also appears to be valid because it was recently found to be useful as a predictor of clinical outcome in colorectal and breast carcinomas (54–56). We also examined cyclin D1 immunostaining. When detectable, cyclin D1 staining was predominantly nuclear and variable in terms of intensity. The normal mucosa sections showed only weak to undetectable signal, and less than 20% of the cells gave detectable staining. For this reason (see also Ref. 7), we scored cyclin D1 immunostaining by the percentage of total cells that were positive and also by the intensity (weak, moderate, or strong) of staining. We considered samples positive if more than 20% of the cells had moderate or strong staining. To assess Ki67 staining, we used the Ki67 score described by Johnston *et al.* (57).

Western and Northern Blot Analyses. Proteins were extracted from the tissues and subjected to Western blot analysis with a $p27^{Kip1}$ antibody as described previously (48). RNA was extracted after solubilizing the tissue in guanidinium thiocyanate solution and then examined by Northern blot analysis as described previously (58). A 1.5-kb human $p27^{Kip1}$ cDNA was used as the probe following random primer labeling with ^{32}P. The histological analysis of frozen sections from the tissues used for the Western and Northern blot studies confirmed the presence of only a minimal stromal component in the samples analyzed.

The intensities of each band on the Western and Northern blots were

quantitated on a Molecular Dynamics computing densitometer (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software, version 3.22.

Statistical Analysis. Statistical analyses were performed using the Instat 2.0 program. The associations between the discrete variables were assessed using the χ^2 and the Fisher exact tests. Mean values were compared using the Mann-Whitney (for two categories) and the Kruskal-Wallis (for three or more categories) tests. Correlations between two parameters were also performed by linear regression and considered statistically significant when the coefficient of determination (r^2 value) was larger than 0.696 (59).

RESULTS

Immunohistochemical Studies on the Expression of p27[Kip1] and Ki67

Normal Mucosa Samples. Among the 20 normal colonic mucosa samples, there was a wide range (5–75%) in the percentage of the epithelial cells that displayed nuclear immunostaining for p27[Kip1], with a mean ± SD of 45.7 ± 18.5% (Fig. 1*a*). The median value was about 50%. Therefore, in our subsequent analyses, samples in which 50% or more of the cells were positive for p27[Kip1] staining were defined as "high expressors," and those with less than 50% were defined as as "low expressors." The 50% cutoff value was also used by previous investigators to distinguish high and low p27[Kip1] expression, and in their studies, it had prognostic significance for cases of colon cancer (52). A typical histological section of normal colonic mucosa is shown in Fig. 2*A*. The positive colonic epithelial cells displayed moderately intense nuclear staining and were distributed throughout the length of the crypts. This included the basal proliferative compartment, as well as the middle and upper thirds of the crypts, which contain differentiated cells. On the other hand, nuclear staining for Ki67 was confined to the cells in the lower one-third of

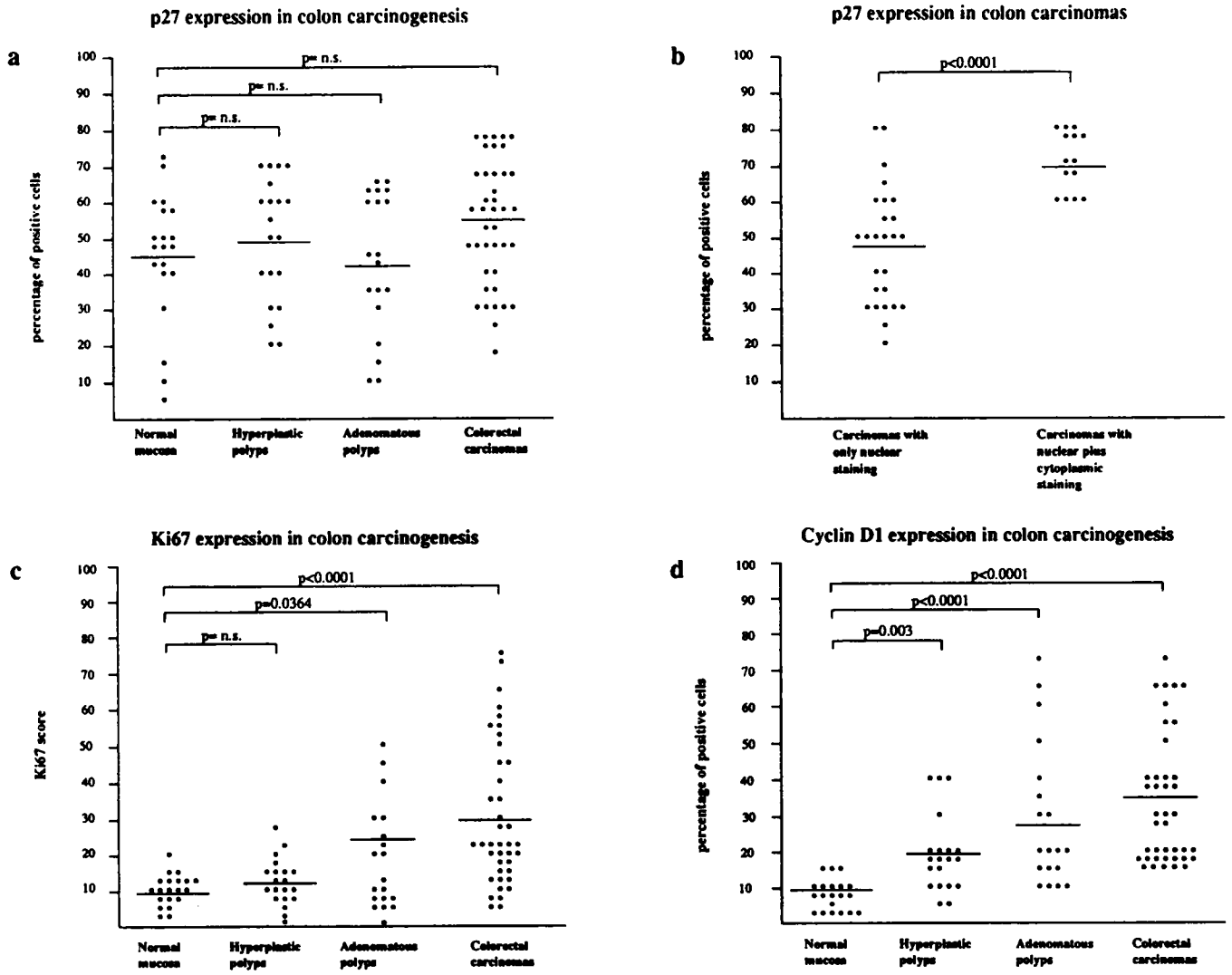

Fig. 1. Immunostaining studies on the expression of p27[Kip1], Ki67 and cyclin D1 in various types of human colon tissues. *a*, p27[Kip1] expression, expressed as percentage of positive cells in normal mucosa, hyperplastic polyps, adenomatous polyps, and colorectal carcinomas. *Horizontal bars*, mean value for each group of samples. *Ps* indicate that there was no significant increase in the mean values between the normal mucosa, the polyps, or the carcinomas. Furthermore, there was no significant increase in these mean values during tumor progression, considering all of the groups ($P = 0.1227$). *b*, p27[Kip1] expression in colorectal carcinomas. The percentage of positive cells is shown for two subsets of carcinomas: one with only nuclear staining and the other with nuclear plus cytoplasmic staining. A significative difference was seen between the mean values for these two groups ($P < 0.0001$). *c*, Ki67 score in normal mucosa, hyperplastic polyps, adenomatous polyps, and colorectal carcinomas. *Horizontal bars*, mean values for each group. *Ps* indicate significant increases in these mean values in the polyps and carcinomas when compared to the normal mucosa samples. Furthermore, a comparison of the mean values for all of these groups of samples showed a significant increase during tumor progression ($P < 0.0001$). *d*, cyclin D1 expression, expressed as percentage of positive cells in normal mucosa, hyperplastic polyps, adenomatous polyps, and colorectal carcinomas. *Horizontal bars* mean values for each group. *Ps* for differences between the normal mucosa and the polyps and carcinomas are shown. A comparison of the mean values across all of these groups of samples showed a significant increase during tumor progression ($P < 0.0001$). For details on the immunostaining methods and scoring procedure, see "Materials and Methods."

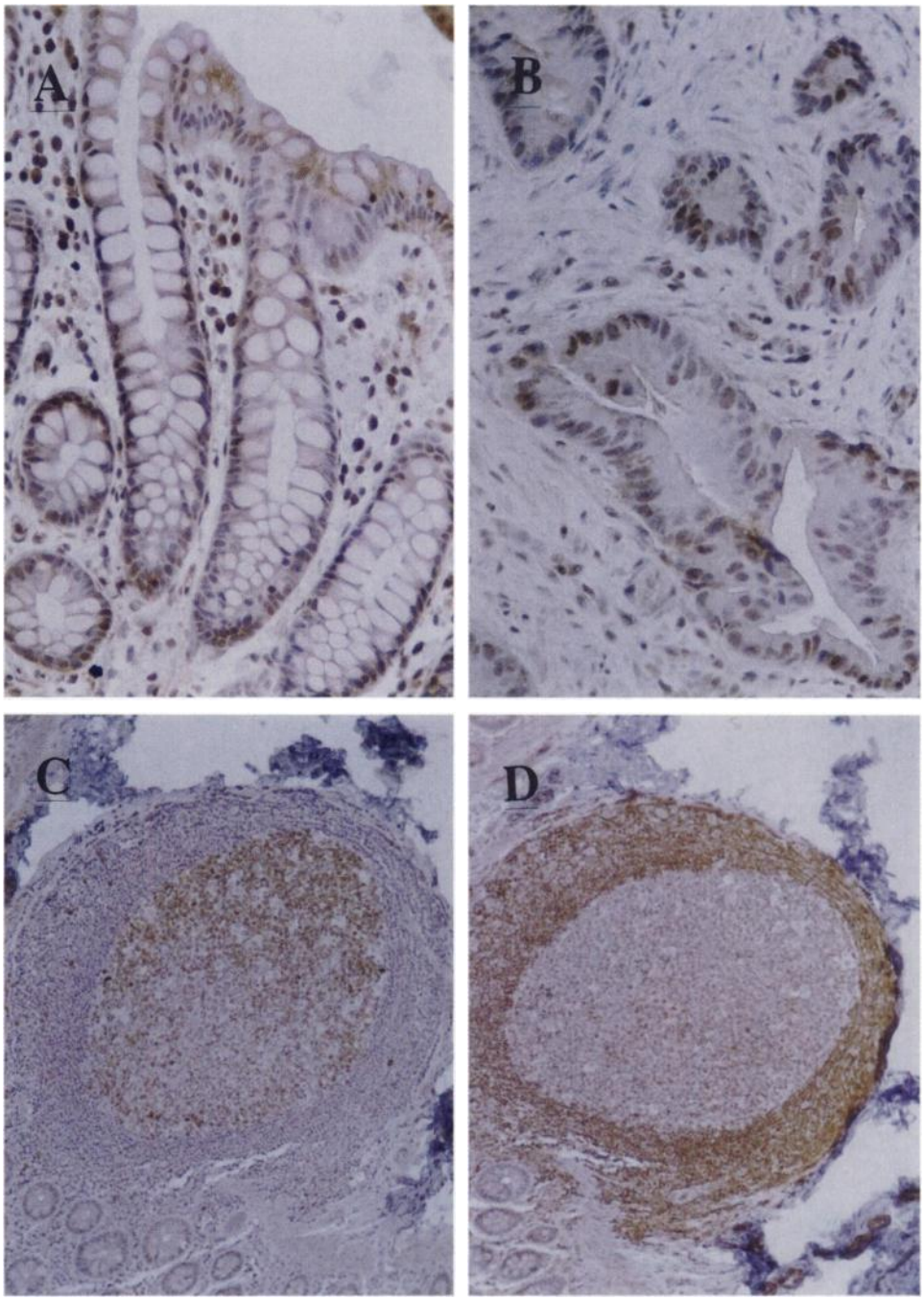

Fig. 2. Representative histological sections demonstrating topology of $p27^{Kip1}$ and Ki67 immunostaining. *A*, normal colonic mucosa. $p27^{Kip1}$ staining is nuclear and is located in cells along the entire length of the crypts, including the basal proliferative region. In the lamina propria, lymphoid cells show very intense staining for $p27^{Kip1}$. ×250. *B*, well differentiated colon carcinoma. This tumor displays numerous cells that have nuclear staining for $p27^{Kip1}$. ×250. *C*, lymphatic follicle in normal colonic mucosa. Ki67 immunostaining is exclusively present in the germinal center of the follicle. ×250. *D*, serial section of the lymphatic follicle shown in *C*. $p27^{Kip1}$ immunostaining is exclusively present in the peripheral, nonproliferating area. ×250. The immunostaining used the avidin-biotin complex technique and 3,3′-diaminobenzidine development (*brown*). Cells were lightly counterstained with hematoxylin. For additional details, see "Materials and Methods."

the crypts (data not shown). The mean value for the percentage of normal colonic cells that were positive for Ki67 was 10.2 ± 3.9% (Fig. 1*c*). The mean Ki67 value for the group of samples that had high $p27^{Kip1}$ expression was essentially the same as that for the low $p27^{Kip1}$ group (10.0 ± 3.1% *versus* 10.9 ± 5.5%). Therefore, by this criterion, as well as by our findings on the regions of the crypts in which $p27^{Kip1}$ was expressed, there was no correlation between extent of cell proliferation and $p27^{Kip1}$ positivity.

A striking finding was the intense staining of lymphoid cells for $p27^{Kip1}$ (Fig. 2*A*). This was also seen in the polyps and carcinomas. Within the lymphatic follicles of the colonic mucosa, the germinal center showed an abundance of Ki67 staining but no $p27^{Kip1}$-positive cells, whereas in the peripheral zone, there was an inverse pattern of staining (Fig. 2, *C* and *D*). Thus, in contrast to the colonic epithelial cells, in the lymphoid cells, the expression of these two proteins was mutually exclusive, suggesting that proliferating lymphoid cells, but not proliferating colonic epithelial cells, markedly down-regulate the expression of $p27^{Kip1}$.

Hyperplastic Polyps. The 20 hyperplastic polyp samples also displayed a wide range of values for percentage of $p27^{Kip1}$-positive cells (20–70%), with a mean value of 49.1 ± 17.4%, similar to that found in the series of normal mucosa samples (Fig. 1*a*). The distribution of $p27^{Kip1}$-positive cells along the length of the crypt was also similar to that seen in the normal mucosa. Ki67 staining was prominent in the lower one-third, and isolated stained cells were observed in the middle one-third of the crypts (data not shown).

Adenomatous Polyps. The 19 adenomas showed a wide range of $p27^{Kip1}$-positive cells with a mean value of 43.4 ± 19.8% (Fig. 1*a*). The cells with $p27^{Kip1}$-positive nuclei were located throughout the lesions, in the superficial epithelium and also along the whole length

of the crypts. Ki67 staining was present in the basal area and extended to the middle and upper thirds of the crypts. Thus, even in the adenomas, $p27^{Kip1}$-positive cells were present in areas of both high and low cell proliferation.

Colorectal Carcinomas. The 40 colorectal carcinomas showed a range of nuclear-positive $p27^{Kip1}$ cells of 10–80%, with a mean value of 55.8 ± 18.3% (Fig. 1*a*). The cells with $p27^{Kip1}$-positive nuclei were distributed through the tumor section. The intensity of staining was generally moderate, with only a few positive tumor cells showing stronger immunoreactivity (Fig. 2*B*). Ki67-positive cells were irregularly scattered throughout pseudoglandular structures. There was not an inverse correlation between regions of high $p27^{Kip1}$ and high Ki67 expression among the tumor cells, nor was there a significant difference between Ki67 mean values between the carcinomas that expressed a high or low percentage of $p27^{Kip1}$-positive nuclei (35.4 ± 15.3% *versus* 26.5% ± 12.4%, respectively).

A subset (35%) of the carcinomas displayed not only nuclear staining for $p27^{Kip1}$ but also diffuse cytoplasmic staining. This cytoplasmic staining was peculiar to the carcinomas because it was not seen in the normal mucosa samples, hyperplastic polyps, or adenomatous polyps. Furthermore, among the group of carcinomas that displayed both nuclear and cytoplasmic staining, the percentage of cells that were positive for $p27^{Kip1}$ was significantly higher than in the group of carcinomas that had only nuclear staining (71.4 ± 8.3% *versus* 47.3 ± 16.5%; $P < 0.0001$; Fig. 1*b*).

Table 1 summarizes possible correlations between the extent of $p27^{Kip1}$ expression among the 40 colorectal carcinomas and various clinical and pathological parameters. As described above (also see "Materials and Methods"), high expressors were defined as tumors in which 50% or more of the cells were positive for $p27^{Kip1}$ staining, and low expressors were defined as those in which this value was less than 50%. We found a significant correlation between carcinomas that had high $p27^{Kip1}$ levels and the well and moderately differentiated tumor grades ($P = 0.0007$; Table 1). Furthermore, $p27^{Kip1}$-positive cells were located predominantly in areas of tumors with low-grade architectural and cytological alterations. The mean values for percentage of positive $p27^{Kip1}$ tumor cells were 63.0 ± 16.2% in the well and moderately differentiated subsets of tumors and 43.7 ± 14.82% in the poorly differentiated subset ($P = 0.0020$). A statistically significant association was also seen between $p27^{Kip1}$ expression and increased tumor size ($P = 0.0292$; Table 1), but the validity of this association is uncertain because of the small number of cases in one group of this contingency category. No other significant association between $p27^{Kip1}$ expression and the other clinicopathological parameters was observed (Table 1). As mentioned above, some of the carcinomas displayed both nuclear and cytoplasmic $p27^{Kip1}$ staining, but the number of these cases was too small to search for possible clinical and pathological correlations unique to this subset. However, we observed that cases with only nuclear staining were more frequently the well and moderately differentiated tumors, as described above, whereas the cases with both nuclear and cytoplasmic staining were equally distributed between the low- and high-grade tumors (data not shown).

Table 1 *p27 expression and clinicopathological parameters*

Statistical analyses were performed by the χ^2 test. $P < 0.05$ was considered significant.

	p27		
	High expressors	Low expressors	*P*
Age (yr)			
<60	8 (20%)	1 (2%)	0.03994
≥60	21 (52%)	10 (25%)	
Sex			
Male	17 (42%)	5 (12%)	0.4977
Female	12 (30%)	6 (15%)	
Tumor site			
Right	10 (25%)	5 (12%)	0.7162
Left	19 (48%)	6 (15%)	
Tumor size			
<2 cm	7 (20%)	6 (15%)	0.0292[a]
2–4 cm	10 (25%)	5 (12%)	
>4 cm	12 (30%)	0 (0%)	
Tumor type			
Adenocarcinoma Nos	23 (58%)	10 (25%)	0.6479
Other	6 (15%)	1 (2%)	
Tumor grade			
Well/moderately differentiated	23 (57%)	2 (5%)	0.0007[c]
Poorly differentiated[b]	6 (15%)	9 (22%)	
Nodal involvement			
Yes	11 (28%)	2 (5%)	0.2859
No	18 (45%)	9 (22%)	
Tumor stage			
A+B	17 (42%)	9 (22%)	0.2698
C+D	12 (30%)	2 (5%)	

[a] The *P* value of this association was not certain because of the presence of a category in which the number of cases was less than 5% of the total number of cases.

[b] Including mucinous, signet ring cell, squamous cell, and undifferentiated carcinomas.

[c] This association was significant even when the tumors were separated into categories with regard to histological grade (well, moderately, and poorly differentiated; $P < 0.0013$). However, in the latter analysis, the presence of a category in which the number of cases was less than 5% of the total number of cases affected the validity of the test.

Immunohistochemical Studies on Cyclin D1 Expression. In view of previous evidence that cyclin D1 is often overexpressed in colorectal carcinomas (7, 8), it was of interest to also evaluate by immunostaining cyclin D1 expression in the above set of samples. The results are summarized in Fig. 1*d*. The normal mucosa samples displayed less than 20% of cyclin D1-positive cells, with a mean value of 9.7 ± 3.9%. The immunostaining for cyclin D1 was generally weak and was mainly nuclear, but some samples also showed cytoplasmic staining, as described previously (8). Positive cells were mainly located in the lower one-third of the crypts but were also present in other regions, including the luminal surface (data not shown). Hyperplastic polyps, adenomas, and carcinomas displayed moderate or intense staining and a higher percentage of positive cells when compared to the normal mucosa samples. Thus, the mean values for percentage of positive cells in the hyperplastic polyps, adenomatous polyps, and colorectal carcinomas were 20.3 ± 10.2%, 28.6 ± 18%, and 34.5 ± 16.1%, respectively (Fig. 1*d*). The differences in the mean values between the normal mucosa and the adenomatous polyps or the colorectal carcinomas were highly significant ($P < 0.0001$ in both cases). There was also a significant increase in the mean values for Ki67 expression between normal mucosa and adenomatous polyps or normal mucosa and colorectal carcinomas ($P = 0.0364$ and $P < 0.0001$, respectively; Fig. 1*c*). However, this was not true for $p27^{Kip1}$ ($P = 0.8111$ and $P = 0.0651$, respectively; Fig. 1*a*). Moreover, an analysis across all categories of tissue samples showed significant increases in the mean values of Ki67 and cyclin D1 during the progressive stages of colon carcinogenesis ($P < 0.001$ in both cases), but this was not true for $p27^{Kip1}$ ($P = 0.1227$).

Among the colorectal carcinomas, we found no correlation between cyclin D1 expression and the various clinical and pathological parameters listed in Table 1 (data not shown). In the series of adenomatous polyps, there was a significant positive association between cyclin D1 and high $p27^{Kip1}$ expression ($P = 0.0408$) by the χ^2 test, but no significant association was seen between these two proteins in the series of colorectal carcinomas using either the χ^2 test ($P = 1.000$) or the linear regression analysis ($r^2 = 0.007$). It is of interest that 8 of the 9 (88%) carcinoma samples that showed high expression of both of these proteins had only nuclear staining for $p27^{Kip1}$, whereas only 1 of these 9 (12%) carcinomas showed nuclear plus cytoplasmic staining for $p27^{Kip1}$ (data not shown). Thus, there is a significant association between increased expression of these two proteins in a subset of the carcinoma samples.

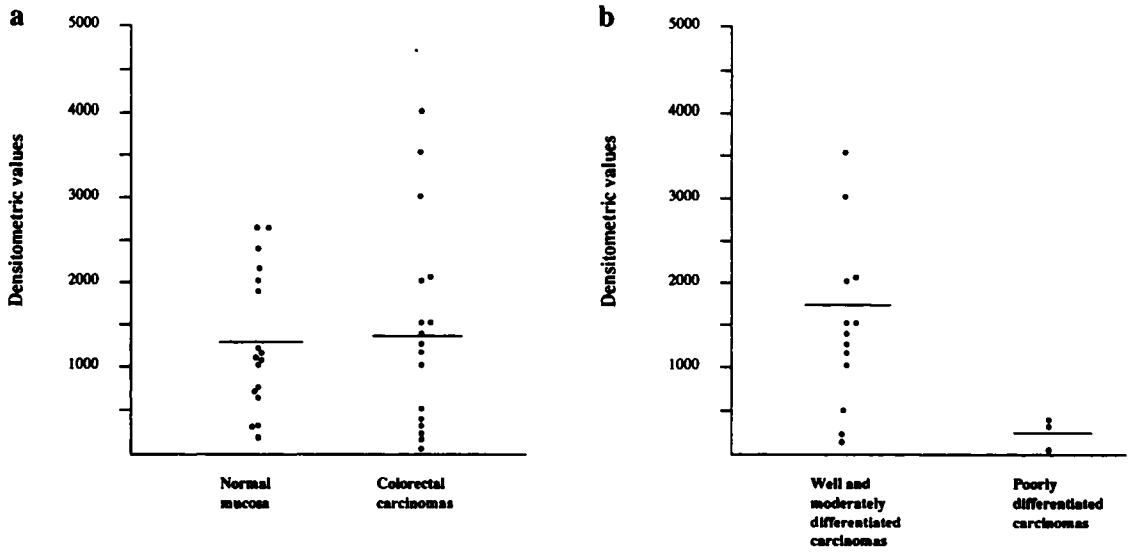

Fig. 3. Summary of Western blot analysis for $p27^{Kip1}$ expression on 17 pairs of colorectal carcinoma and normal colonic mucosa samples. *a*, comparison of $p27^{Kip1}$ protein levels between normal mucosa and carcinomas. The $p27^{Kip1}$ values were obtained by densitometric scanning of the Western blot. *Bars*, mean values. A comparison of the mean values between these two groups did not show a statistically significant difference ($P = 0.8220$). *b*, comparison of $p27^{Kip1}$ protein levels between well and moderately differentiated and poorly differentiated carcinomas. The mean value of the former group is significantly higher than that of the latter group ($P = 0.0324$). For additional details, see "Materials and Methods."

Western and Northern Blot Analyses of $p27^{Kip1}$ Expression. To extend the results obtained by immunohistochemical analysis of histological sections, we also examined by Western blot analysis an additional 17 primary colorectal carcinoma surgical specimens, together with paired adjacent normal colonic mucosa samples obtained from the same cases, for levels of expression of $p27^{Kip1}$ in total tissue lysates. The results obtained were quantitated by densitometry. As in the immunostaining studies, both the normal colonic mucosa and carcinoma samples displayed a wide range of expression of the $p27^{Kip1}$ protein but similar mean values (Fig. 3*a*). Seven of the 17 carcinomas (41%) had higher levels of the $p27^{Kip1}$ protein than their corresponding paired adjacent normal mucosa samples. The remaining carcinoma samples had levels of this protein that were equal to or less than those in the paired normal mucosa sample.

In view of the results obtained with $p27^{Kip1}$ immunostaining (Fig. 1*b*), we also analyzed these Western blot data for possible correlations with the histological grade of the carcinomas. Again, we found a correlation between high $p27^{Kip1}$ protein expression and carcinomas that were well or moderately differentiated (Fig. 3*b*). Thus, the mean densitometric value for the level of the $p27^{Kip1}$ protein in the well and moderately differentiated carcinomas was significantly higher than that of the poorly differentiated carcinomas (1711 ± 1078 *versus* 315 ± 177; $P = 0.0324$; Fig. 3*b*). We also performed Northern blot analysis on mRNA extracts prepared from seven of these paired normal mucosa and carcinoma samples. All of the samples revealed a characteristic 2.5-kb $p27^{Kip1}$ mRNA band. In four (56%) cases, the level of this mRNA was 1.8–15.6-fold higher in the carcinoma sample than in the paired adjacent normal mucosa. A comparison between Western and Northern blot analyses revealed that there was no consistent correlation between the $p27^{Kip1}$ protein level and mRNA levels in each sample (data not shown).

DISCUSSION

The present study indicates that in normal human colonic mucosa and during colorectal carcinogenesis, $p27^{Kip1}$ expression does not correlate with cell proliferation. Thus, our immunostaining studies indicated that in normal colonic mucosa, $p27^{Kip1}$ was expressed in both the basal proliferative region of the crypts and in the differentiated upper regions of the crypts (Fig. 2*A*), in contrast to Ki67, which was expressed only in the basal region of the crypts. Although a series of hyperplastic polyps, adenomatous polyps, and colorectal carcinoma samples displayed a progressive increase in the mean score for Ki67 expression, there was no significant upward or downward trend in the respective values for $p27^{Kip1}$ expression (Fig. 1, *c* and *a*, respectively). In addition, among the colorectal carcinomas, there was no correlation between $p27^{Kip1}$ and Ki67 expression. The absence of an inverse correlation between $p27^{Kip1}$ expression and extent of cell proliferation is somewhat surprising in view of the known role of this protein as a negative regulator of the cell cycle (25). This finding is, however, consistent with our previous evidence that $p27^{Kip1}$ is often expressed at relatively high levels in exponentially growing human cancer cell lines (46–50). At the same time, we did find that well and moderately differentiated colorectal carcinomas had higher mean values for $p27^{Kip1}$ than poorly differentiated carcinomas (Table 1). Western blot analyses for levels of expression of the $p27^{Kip1}$ protein done on 17 colorectal carcinoma samples and paired adjacent normal mucosa samples also indicated no consistent increase or decrease in the tumor *versus* the normal samples and also confirmed the association of high expression in the low-grade carcinomas (Fig. 3*b*).

During the course of our studies, other investigators have reported findings on $p27^{Kip1}$ expression in human colon, breast, and non-small cell lung carcinomas (54–56, 60, 61) and gastric carcinomas[4] that are consistent with our results. These studies also found no correlation between the level of $p27^{Kip1}$ expression in tumors and the extent of cell proliferation, but there was a positive correlation between high $p27^{Kip1}$ expression and low-grade histology and/or a more favorable prognosis. The previous studies on colon cancer (54, 60) did not examine colonic polyps or paired colorectal carcinoma and adjacent normal mucosa samples. In a subset of our paired samples, we also did Northern blot analyses for $p27^{Kip1}$ mRNA and found no consistent correlation between the level of $p27^{Kip1}$ mRNA and protein in the

[4] Yasui, W., Kudo, Y., Semba, S., Yokozaki, H., and Tahara, E. Reduced expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$ is associated with advanced stage and invasiveness of gastric carcinomas. Jpn. J. Cancer Res, *88:* 625–629, 1997.

same sample. This finding is consistent with evidence that cellular levels of this protein are regulated mainly via posttranscriptional mechanisms, including ubiquitin-proteasome-mediated proteolysis (41, 42, 54). Indeed, there is evidence that the low levels of $p27^{Kip1}$ seen in some colon and lung carcinomas are due to increased activity of this proteolytic mechanism (54, 61).

Thirty-five % of the colorectal carcinomas displayed not only nuclear immunostaining for $p27^{Kip1}$ but also diffuse cytoplasmic staining. This group of carcinomas also displayed a higher percentage of cells that were positive for nuclear $p27^{Kip1}$ staining (Fig. 1*b*). At the present time, it is not clear whether this represents simply leakage of the protein from the nucleus or proteolytic degradation. Alternatively, because there is evidence that $p27^{Kip1}$ and $p21^{Cip1}$ direct the accumulation of cyclin D1 to the nucleus (61), the presence of $p27^{Kip1}$ in the cytoplasm may reflect a defect in nuclear translocation. Other investigators have also noted cytoplasmic immunostaining for $p27^{Kip1}$ in some tumor samples (54). *In vitro* studies in colon cancer cell lines may clarify the significance of this finding.

In this study, we did not examine the level of expression of another CKI $p21^{waf1}$, but other investigators have studied the expression of this protein in normal human colonic mucosa and during colorectal carcinogenesis. In the normal colonic mucosa, $p21^{waf1}$ protein (62, 63) and mRNA (64) are expressed mainly in the upper one-third of the crypts and the superficial differentiated cells and not in the Ki67-positive basal proliferative compartment. This is in contrast to our findings in the present study, in which $p27^{Kip1}$ expression did not show a reciprocal relationship with Ki67 expression in normal colonic mucosa. In the previous studies on colorectal carcinomas, $p21^{waf1}$ expression was heterogeneous. High expression was associated with lower stage tumors and an apparently normal *p53* gene, whereas low expression appeared to be associated with mutations in the *p53* gene. However, some tumors displayed high levels of $p21^{waf1}$ even in the presence of presumed mutations in p53 (63, 65). It appears that both precursor lesions and carcinomas can display dysregulation in the expression of $p21^{waf1}$ (62, 65). We are not aware of any studies on the prognostic significance of $p21^{waf1}$, although its normal association with differentiation and its loss in high-grade tumors suggest that loss of $p21^{waf1}$ expression may be associated with a poor prognosis.

We have previously reported increased expression of cyclin D1 in human adenomatous polyps of the colon and in colon carcinomas (8). Recent studies indicate that this is also seen in the small polyps obtained from patients with polyposis coli or APC-deficient mice with polyposis coli (11). Therefore, increased expression of cyclin D1 can occur at an early stage of colon carcinogenesis. The present study confirms and extends these findings. Thus, in contrast to $p27^{Kip1}$ (Fig. 1*a*), normal colonic mucosa expressed very low levels of cyclin D1, and there was a progressive increase in the expression of this protein in colonic polyps and colorectal carcinomas (Fig. 1*d*). There was a significant correlation between high expression of $p27^{Kip1}$ and cyclin D1 in the adenomatous polyps and in the subset of carcinomas that had only nuclear $p27^{Kip1}$ expression. This finding is reminiscent of our evidence that these two proteins appear to be coregulated in a subset of human cancer cell lines (46, 47, 50). A recent study (60) also found an association between levels of expression of cyclin D1 and $p27^{Kip1}$ in primary human breast cancers. This association has also been seen not only in esophageal cancer cell lines (46, 66) but also in primary squamous carcinomas of the esophagus.[5]

During the course of these studies, we observed that lymphocytes in the stroma of the normal colon mucosa and in the polyp and carcinoma samples displayed intense immunostaining for $p27^{Kip1}$. Within lymphatic follicles, the germinal center showed intense Ki67 staining but no $p27^{Kip1}$ staining, whereas in the peripheral zone, there was an inverse staining pattern (Fig. 2, *C* and *D*). Therefore, in contrast to normal colonic mucosa and colon carcinoma cells, proliferating lymphoid cells markedly down-regulate the expression of $p27^{Kip1}$. Furthermore, the intensity of $p27^{Kip1}$ staining in the lymphatic cells was usually much greater than in the positive colonic mucosal cells. Therefore, it would appear that the role of $p27^{Kip1}$ in regulating withdrawal from the cell cycle varies between cell types. This conclusion is consistent with effects seen in control and $p27^{Kip1}$-deficient mice (63, 64). In control mice, among various normal tissues, the thymus and spleen express the highest level of $p27^{Kip1}$, and in the deficient mice, these organs show the greatest increase in size.

Finally, it is of interest to consider the findings in this study and in other studies on $p27^{Kip1}$ expression in human cancers (54–56, 60, 61) within the broad context of growth control and carcinogenesis. What is the explanation for the apparent paradox (see Fig. 1*a*) that about 50% of adenomatous polyps and colorectal carcinomas of the colon express levels of an inhibitor of the cell cycle that are equal to or greater than those seen in normal colonic mucosa? Other recent studies have also seen relatively high levels of expression of $p27^{Kip1}$ in a subset of colorectal and breast cancers (54–56, 60). In addition, as mentioned above, exponentially dividing cultures of human colon and breast cancer cell lines also frequently express high levels of this protein (46–50, 60). It is curious that another member of the Cip family of CKIs, $p21^{Cip1}$, is also expressed at relatively high levels in some human cancers (67). On the basis of our *in vitro* studies with cell lines, we previously postulated the existence of feedback inhibitory loops between cyclin D or E and $p27^{Kip1}$, the function of which is to maintain homeostatic control between positive- and negative-acting factors involved in cell cycle progression (46–50, 52). The association between high levels of cyclin D1 and $p27^{Kip1}$ in adenomatous polyps and a subset of colon carcinomas seen in the present study, and also in previous studies on breast (60) and esophageal carcinomas (see above), is consistent with this hypothesis. This association was not, however, seen in all of the carcinomas, presumably because cyclin E (48–50) and other unknown factors can also influence the level of $p27^{Kip1}$ expression. The function of $p27^{Kip1}$ in the above-described homeostatic feedback loop might be to prevent potentially toxic effects of excessive cyclin D/Cdk or cyclin E/Cdk kinase activity. The coordinate expression of cyclin D1 and $p27^{Kip1}$ might also be relevant to recent evidence that at low concentrations, members of the Cip family of CKIs may play a positive role by promoting the association of D-type cyclins with Cdk4, thus enhancing kinase activity, and they may also play a role in targeting cyclin D1 and Cdk4 to the nucleus (68).

The association of high $p27^{Kip1}$ expression with low-grade tumors in this and in previously published studies (55, 60, 61) is consistent with evidence for a role of $p27^{Kip1}$ in development and differentiation (40, 69–71), but the precise role that it plays is not apparent at the present time. We do not yet have follow-up data on the cases examined in this study, but other recent studies found that cases of colon, breast, or non-small cell lung cancer with low or absent $p27^{Kip1}$ expression had a poor prognosis (54–56, 61). Perhaps this is because they have escaped the above-mentioned putative homeostatic feedback inhibitory function of $p27^{Kip1}$ through further alterations in the complex circuitry that controls the cell cycle. Additional studies are required to elucidate the multiple factors that influence the levels of $p27^{Kip1}$ expression in human cancer and whether the levels of expression of this protein, cyclin D1, or related cell cycle control proteins can be exploited in the therapy of specific cancers.

[5] Y. Doki, personal communication.

ACKNOWLEDGMENTS

The authors thank W-Q. Xing and E. Okin for technical assistance and J-W. Soh for valuable computer expertise. We are also grateful to Dr. E. Bagella for statistical advice and to B. Castro and P. Jean-Louis for excellent assistance in the preparation of the manuscript.

REFERENCES

1. Hunter, T., and Pines, J. Cyclins and cancer II: cyclin D and CDK inhibitors come of age. Cell, *79:* 573–582, 1994
2. Hall, M., and Peters, G. Genetic alterations of cyclins, cyclin dependent kinases, and Cdk inhibitors in human cancer. Adv. Cancer Res., *68:* 67–108, 1996.
3. Weinstein, I. B., and Zhou, P. Cell cycle control gene defects and human cancer. *In:* J. R. Bertino (ed.), Encyclopedia of Cancer, Vol. 1, pp. 256–267. New York: Academic Press, 1996.
4. Jiang, W., Zhang, Y-J., Kahn, S. M., Hollestein, M. C., Santella, R. M., Lu, S. H., Harris, C. C., Montesano, R., and Weinstein, I. B. Altered expression of the cyclin D1 and retinoblastoma genes in human esophageal cancer. Proc. Natl. Acad. Sci. USA, *90:* 9026–9030, 1993.
5. Zhang, Y-J., Jiang, W., Chen, C. J., Lee, C. S., Kahn, S. M., Santella, R. M., and Weinstein, I. B. Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma. Biochem. Biophys. Res. Commun., *196:* 1010–1016, 1993.
6. Gillett, C., Fantl, V., Smith, R., Fisher, C., Bartek, J., Dickson, C., Barnes, D., and Peters, G. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res., *54:* 1812–1817, 1994.
7. Bartkova, J., Lukas, J., Strauss, M., and Bartek, J. The PRAD-1/cyclin D1 oncogene product accumulates aberrantly in a subset of colorectal carcinomas. Int. J. Cancer, *58:* 568–573, 1994.
8. Arber, N., Hibshoosh, H., Moss, S. F., Sutter, T., Zhang, Y., Begg, M., Wang, S., Weinstein, I. B., and Holt, P. R. Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology, *110:* 669–674, 1996.
9. Keyomarsi, K., Conte, D., Jr., Toyofuku, W., and Fox, M. P. Deregulation of cyclin E in breast cancer. Oncogene, *11:* 941–950, 1995.
10. Khatib, Z. A., Matsushime, H., Valentine, M., Shapiro, D. N., Sherr, C. J., and Look, A. T. Coamplification of the *CDK4* gene with MDM2 and GLI in human sarcomas. Cancer Res., *53:* 5535–5541, 1993.
11. Zhang, T., Nanney, L. B., Luongo, C., Lamps, L., Heppner, K. J., DuBois, R. N., and Beauchamp, R. D. Concurrent overexpression of cyclin D1 and cyclin-dependant kinase 4 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia mice and human familial adenomatous polyposis patients. Cancer Res., *57:* 169–175, 1997.
12. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature (Lond.), *366:* 704–707, 1993.
13. Hannon, G. J., and Beach, D. $P15^{INK4B}$ is a potential effector of TGF-β-induced cell cycle arrest. Nature (Lond.), *371:* 257–261, 1994.
14. Guan, K-L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. Growth suppression by p18, a $p16^{INK4/MTS1}$ and $p14^{INK4/MTS2}$-related CDK6 inhibitor, correlates with wild-type Rb function. Genes Dev., *8:* 2939–2952, 1994.
15. Hirai, H., Roussel, M. F., Kato, J., Ashmun, R. A., and Sherr, C. J. Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell. Biol., *15:* 2672–2681, 1995.
16. Tam, S. W., Shay, J. W., and Pagano, M. Differential expression and cell cycle regulation of the cyclin dependent kinase 4 inhibitor p16/INK4. Cancer Res., *54:* 5816–5820, 1994.
17. Jin, X., Nguyen, D., Zhang, D., Kyritsis, A. P., and Roth, J. A. Cell cycle arrest and inhibition of tumor cell proliferation by the p16/INK4 gene mediated by an adenovirus vector. Cancer Res., *55:* 3250–3253, 1995.
18. Reed, J. A., Loganzo, F. j., Shea, C. R., Walker, G. J., Flores, J. F., Glendening, J. M., Bogdany, J. K., Shiel, M. J., Haluska, F. J., and Fountain, J. W. Loss of expression of the p16/cyclin-dependent kinase inhibitor 2 tumor suppressor gene in melanocytic lesions correlates with invasive stage of tumor progression. Cancer Res., *55:* 2713–2718, 1995.
19. Sill, H., Goldman, J. M., and Cross, N. Homozygous deletions of the *p16* tumor suppressor gene are associated with lymphoid transformation of chronic myeloid leukemia. Blood, *85:* 2013–2016, 1995.
20. Hussussian, C. J., Struewing, J. P., Goldstein, A. M., Higgins, P. A. T., Ally, D. S., Sheahan, M. D., Clark, W. H. J., Tucker, M. A., and Dracopoli, N. M. Germline p16 mutations in familial melanoma. Nat. Genet., *8:* 15–21, 1994.
21. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumor suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med., *1:* 686–692, 1995.
22. Serrano, M., Lee, H-W., Chin, L., Cordon-Cardo, C., Beach, D., and R. A., D. Role of the *INK4a* locus in tumor suppression and cell mortality. Cell, *85:* 27–37, 1996.
23. Xiong, Y., Hannon, G. J., Zhang, G. J., Casso, D., Kobayashi, R., and Beach, D. p21 is a universal inhibitor of cyclin kinases. Nature (Lond.), *366:* 701–704, 1993.
24. Harper, J. W., Adami, G., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21kd Cdk interacting protein Cip1 is a potent inhibitor of G_1 cyclin-dependent kinases. Cell, *75:* 805–816, 1993.
25. Polyak, K., Lee, M-H., Erdjument-Bromage, A., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. Cloning of $p27^{KIP1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell, *78:* 59–66, 1994.
26. Polyak, K., Kato, J-Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., and Koff, A. $p27^{Kip1}$, a cyclin-Cdk inhibitor, links transforming growth factor-β and contact inhibition to cell cycle arrest. Genes Dev., *8:* 9–22, 1994.
27. Matsuoka, S., Edwards, M., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W., and Elledge, S. J. $p57^{Kip2}$, a structurally distinct member of the Cip1 cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev., *9:* 650–662, 1995.
28. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. Cell, *75:* 817–825, 1993.
29. Michieli, P., Chedid, M., Lin, D., Pierce, J. H., Mercer, W. E., and Givol, D. Induction of WAF1/CIP by a p53 independent pathway. Cancer Res., *54:* 3391–3395, 1994.
30. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. Exp. Cell Res., *211:* 90–98, 1994.
31. Shiohara, M., El-Deiry, W. S., Wada, M., Nakamaki, T., Takeuchi, S., Yang, R., Chen, D-L., Vogelstein, B., and Koeffler, H. P. Absence of WAF1 mutations in a variety of human malignancies. Blood, *84:* 3781–3784, 1994.
32. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. Mice lacking $p21^{CIP1/WAF1}$ undergo normal development but are defective in G_1 checkpoint control. Cell, *82:* 675–684, 1995.
33. Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature (Lond.), *377:* 552–557, 1995.
34. Toyoshima, H., and Hunter, T. p27, a novel inhibitor of G_1 cyclin-Cdk protein kinase activity, is related to p21. Cell, *78:* 67–74, 1994.
35. Kato, J-Y., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. J. Cyclic AMP-induced G_1 phase arrest mediated by an inhibitor ($p27^{KIP1}$) of cyclin-dependent kinase 4 activation. Cell, *79:* 487–496, 1994.
36. Nourse, J., Firpo, E., Flanagan, M. W., Coats, S., Polyak, K., Lee, M-H., Massague, J., Crabtree, G. R., and Roberts, J. M. Interleukin-2-mediated elimination of the $p27^{Kip1}$ cyclin-dependent kinase inhibitor prevented by rapamycin. Nature (Lond.), *372:* 570–573, 1994.
37. Harvat, L. B., Seth, P., and Jetten, A. M. The role of $p27^{Kip1}$ in γ interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. Oncogene, *14:* 2111–2122, 1997.
38. Kuniyasu, H., Yasui, W., Kitahara, K., Naka, K., Yokozaki, H., Akama, Y., Hamamoto, T., Tahara, H., and Tahara, E. Growth inhibitory effect of interferon-β is associated with the induction of cyclin dependent kinase inhibitor $p27^{Kip1}$ in a human gastric cell line. Cell Growth Differ., *8:* 47–52, 1997.
39. Rivard, N., L'Alleimain, G., Bartek, J., and Pouyssegur, J. Abrogation of $p27^{Kip1}$ by cDNA antisense suppresses quiescence (Go state) in fibroblasts. J. Biol. Chem., *271:* 18337–18341, 1996.
40. Kranenburg, O., Scharnhorst, V., Van der Eb, A., and Zantema, A. Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. J. Cell Biol., *131:* 227–234, 1995.
41. Hengst, L., and Reed, S. I. Translational control of $p27^{Kip1}$ accumulation during the cell cycle. Science (Washington DC), *271:* 1861–1864, 1996.
42. Pagano, M., Tam, S. W., Theodoras, A. M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P. R., Draetta, G. F., and Rolfe, M. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science (Washington DC), *269:* 682–685, 1995.
43. Pietenpol, J. A., Bohlander, S. K., Sato, Y., Papadopoulos, N., Liu, B., Friedman, C., Trask, B. J., Roberts, J. M., Kinzler, K. W., Rowley, J. D., and Vogelstein, B. Assignment of the human *p27Kip1* gene to 12p13 and its analysis in leukemias. Cancer Res., *55:* 1206–1210, 1995.
44. Bullrich, F., MacLachlan, T. K., Sang, N., Druck, T., Veronese, M. L., Allen, S. L., Chiorazzi, N., Koff, A., Heubner, K., Croce, C. M., and Giordano, A. Chromosomal mapping of members of the cdc2 family of protein kinases, cdk3, cdk6, PISSLRE, and PITALRE, and a cdk inhibitor, $p27^{Kip1}$, to regions involved in human cancer. Cancer Res., *55:* 1199–1205, 1995.
45. Ponce-Castaneda, M. V., Lee, M-H., Latres, E., Polyak, K., Lacombe, L., Montgomery, K., Mathew, S., Krauter, K., Sheinfeld, J., Massague, J., and Cordon-Cardo, C. $p27^{Kip1}$: chromosomal mapping to 12p12–12p13.1 and absence of mutations in human tumors. Cancer Res., *55:* 1211–1214, 1995.
46. Doki, D., Imoto, M., Han, E. K-H., Sgambato, A., and Weinstein, I. B. Correlations between the levels of expression of cyclin D1 and other cell cycle-related genes in human esophageal cancer cell lines. Carcinogenesis (Lond.), *18:* 1139–1148, 1997.
47. Han, E. K-H., Begemann, M., Sgambato, A., Soh, J-W., Doki, Y., Xing, W-Q., Liu, W., and Weinstein, I. B. Increased expression of cyclin D1 in a murine mammary epithelial cell line induces $p27^{Kip1}$, inhibits growth, and enhances apoptosis. Cell Growth Differ., *7:* 699–710, 1996.
48. Sgambato, A., Han, E. K-H., Zhou, P., Schieren, I., and Weinstein, I. B. Overexpression of cyclin E in the HC11 mouse mammary cell line is associated with growth inhibition and increased expression of $p27^{Kip1}$. Cancer Res., *56:* 1389–1399, 1996.
49. Sgambato, A., Doki, Y., Schieren, I., and Weinstein, I. B. Effects of cyclin E overexpression on cell growth and response to TGF-β depend on cell context and $p27^{Kip1}$ expression. Cell Growth Differ., *8:* 393–405, 1997.
50. Sgambato, A., Zhang, Y-J., Arber, N., Hibshoosh, H., Doki, Y., Ciaparrone, M., Santella, R. M., and Weinstein, I. B. Deregulated expression of p27Kip1 in Human Breast Cancers. Clin. Cancer Res., *3:* 1879–1887, 1997.
51. Zhou, P., Jiang, W., Zhang, Y., Kahn, S. M., Schieren, I., Santella, R. M., and Weinstein, I. B. Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. Oncogene, *11:* 571–580, 1995.
52. Arber, N., Doki, Y., Han, E. K-H., Sgambato, A., Zhou, P., Kim, N., Delohery, T., Klein, M. G., Holt, P. R., and Weinstein, I. B. Antisense to cyclin D1 inhibits the

growth and tumorigenicity of human colon cancer cells. Cancer Res., *57:* 1569–1574, 1997.

53. Gerdes, J., Schwab, U., Lenke, H., and Stein, H. Production of a monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. Int. J. Cancer, *31:* 13–20, 1983.
54. Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, G. M., and Pagano, M. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nat. Med., *3:* 231–234, 1997.
55. Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., Franssen, E., Pritchard, K. I., and Slingerland, J. M. Increased level of the cell-cycle inhibitor p27^{Kip1} protein: prognostic implications in primary breast cancer. Nat. Med., *3:* 227–230, 1997.
56. Porter, P. L., Malone, K. E., Heagerty, P. J., Alexander, G. M., Gatti, L. A., Firpo, E. J., Daling, J. R., and Roberts, J. M. Expression of cell-cycle regulators p27^{Kip1} and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nat. Med., *3:* 222–225, 1997.
57. Johnston, P. G., O'Brien, M. J., Dervan, P. A., and Carney, D. N. Immunohistochemical analysis of cell kinetic parameters in colonic adenocarcinomas, adenomas, and normal mucosa. Hum. Pathol., *7:* 696–700, 1989.
58. Han, E. K., Sgambato, A., Jiang, W., Zhang, Y., Santella, R. M., Doki, Y., Cacace, A. M., Schieren, I., and Weinstein, I. B. Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth. Oncogene, *10:* 953–962, 1995.
59. Zhar, J. H. Biostatistical Analysis. Englewood Cliffs, NJ: Prentice-Hall, Inc., 1974.
60. Fredersdorf, S., Burnes, J., Ailine, A. M., Packham, G., Fallis, L., Gillett, C. E., Royds, J. A., Peston, D., Hall, P. A., Hanby, A. M., Barnes, D. M., Shousha, S., O'Hare, J. M., and Lu, X. High level expression of p27^{Kip1} and cyclin D1 in some human breast cancer cells: inverse correlation between the expression of p27^{Kip1} and degree of malignancy in human breast and colorectal cancers. Proc. Natl. Acad. Sci. USA, *94:* 6380–6385, 1997.
61. Esposito, V., Baldi, A., De Luca, A., Groger, A. M., Loda, M., Giordano, G. G., Caputi, M., Baldi, F., Pagano, M., and Giordano, A. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. Cancer Res., *57:* 3381–3385, 1997.
62. Polyak, K., Hamilton, S. R., Vogelstein, B., and Kinsler, K. W. Early alteration of cell-cycle-regulated gene expression in colorectal neoplasia. Am. J. Pathol., *149:* 381–387, 1996.
63. Doglioni, C., Pelosio, P., Laurino, L., Macri, E., Meggiolaro, E., Favretti, F., and Barbareschi, M. p21$^{WAF1/CIP1}$ expression in normal mucosa and in adenomas and adenocarcinomas of the colon: its relationship with differentiation. J. Pathol., *179:* 248–253, 1996.
64. Matushita, K., Kobayashi, S., Kato, M., Itoh, Y., Okuyama, K., Sakiyama, S., and Isono, K. Reduced messenger RNA expression level of p21^{CIP1} in human colorectal carcinoma tissues and its association with p53 gene mutation. Int. J. Cancer, *69:* 259–264, 1996.
65. Sasaki, K., Sato, T., Kurose, A., and Ikeda, E. Immunohistochemical detection of p21$^{WAF1/CIP1SDI1}$ and p53 proteins in formalin-fixed, paraffin-embedded tissue sections of colorectal carcinoma. Hum. Pathol., *27:* 912–916, 1996.
66. Kitahara, K., Yasui, W., Yokozaki, H., Semba, S., Hamamoto, T., Hisatsugu, T., and Tahara, E. Expression of cyclin D1, CDK4 and p27^{Kip1} is associated with the p16^{MST1} gene status in human esophageal carcinoma cell lines. J. Exp. Ther. Oncol., *1:* 7–12, 1996.
67. Barbareschi, M., Caffo, O., Doglioni, C., Fina, P., Marchetti, A., Buttitta, F., Leek, R., Morelli, L., Leonardi, F., Bevilacqua, G., Galla Palma, P., and Harris, A. L. p21^{WAF1} immunohistochemical expression in breast carcinomas: correlations with clinicopathological data, oestrogen receptor status MIB1 expression, *p53* gene and protein alterations and relapse-free survival. Br. J. Cancer, *74:* 208–215, 1996.
68. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. New functional activities for the p21 family of CDK inhibitors. Genes Dev., *11:* 847–862, 1997.
69. Kiyokawa, H., Kineman, R. D., Manova-Todorova, K. O., Soares, V. C., Hoffman, E. S., Ono, M., Khanam, D., Hayday, A. C., Frohman, L. A., and Koff, A. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27^{Kip1}. Cell, *85:* 721–732, 1996.
70. Fero, M. L., Rivkin, M., Tasch, M., Porter, P., Carow, C. E., Firpo, E., Polyak, K., Tsai, L-H., Broudy, V., Perlmutter, R. M., Kaushansky, K., and Roberts, J. M. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27^{Kip1}-deficient mice. Cell, *85:* 733–744, 1996.
71. Hauser, P. J., Agrawal, D., Flanagan, M., and Pledge, W. J. The role of p27^{Kip1} in the *in vitro* differentiation of murine keratinocytes. Cell Growth Differ., *8:* 203–211, 1997.