

Endocrine dysfunction in p27^{Kip1} deficient mice and susceptibility to *Wnt-1* driven breast cancer

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The cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} (p27) is a marker of prognosis in many cancers, including breast cancer. Low p27 expression correlates with poor prognosis, especially in hormone receptor positive breast tumors. This association suggests a role for p27 in hormone-dependent cancer. We used the *Wnt-1* transgenic mouse model to further explore the role of p27 in hormone-driven breast cancer. We found that p27 deficiency did not alter breast cancer rate in either male or female *Wnt-1* mice. However, we did find p27^{-/-} females had reduced levels of serum progesterone (P) and increased variability in estradiol (E), which could have affected their cancer susceptibility. To equalize hormone levels, an additional cohort of *Wnt-1* female mice was ovariectomized and implanted with slow release pellets of E and P. Although this treatment did not alter the breast cancer rate, it did accelerate the development of pituitary and gastric tumors in p27^{-/-} mice. This study shows that while not a significant inhibitor of *Wnt-1*-driven breast cancer, p27 inhibits gastric tumors, whose latency is modulated by sex steroids.

Introduction

The cyclin-dependent kinase (Cdk) inhibitor p27^{Kip1} (p27) regulates the G₀ to S cell cycle transition by binding to and inhibiting the activity of Cdks (1). Many extracellular signals that increase or decrease cell proliferation act by decreasing or increasing p27 levels, respectively. Reduced expression of p27 is frequently observed in human cancers and this correlates with poor prognosis (2). Notable examples include cancers of the lung, colon, prostate and breast. In breast cancer, where p27 has been extensively studied, 11 of 17 studies show reduced p27 correlates significantly with poor prognosis in multivariate analysis (2). Reduced p27 increased the risk of relapse or death 1.3- to 4-fold over 5–17 years of follow up.

Porter *et al.* (3) recently reported results from a large study of >3000 breast cancer patients, all of whom received similar adjuvant chemotherapy, confirming that p27 levels were prognostic, when controlled for treatment. This association was strongest in steroid receptor positive, tamoxifen-treated patients. In addition, high levels of p27 were strongly associated with positive estrogen receptor (ER) and progesterone receptor status and were an independent predictor of response to hormonal therapy (4). Ovarian hormones, notably estradiol (E) and progesterone (P) play a major role in the development of breast cancer (5). Over 70% of human breast cancers are ER positive and show estrogen-dependent growth. One proposed mechanism of estrogen-stimulated breast cancer growth is through the regulation of p27. Estrogen-treated MCF-7 breast cancer cells show a decrease in p27 and increased cyclinE/Cdk2 activity and G₁-S transition (6). Conversely, levels of p27 increase in tamoxifen-treated ER-positive breast cancer cells and p27 was required for subsequent cell cycle

arrest (7). Together, these studies suggest p27 may provide a causal link between steroid hormones and breast cancer.

Despite the accepted role of p27 as a cell cycle inhibitor and the correlation of reduced expression with tumor aggressiveness, mutations in the *CDKN1B* gene encoding p27 are rarely observed in human tumors (8–10). A causal role for p27 in tumor suppression was demonstrated by the tumor predisposition of p27-deficient mice (11). In susceptible genetic backgrounds or after exposure to carcinogens, both p27-null and p27 heterozygous knockout mice exhibit increased susceptibility to tumor development in multiple tissues, including pituitary (12–14), lung, small intestine, colon (11), lymphoma (15–18), prostate (19) and bladder (20). Recently, germline mutations in the *CDKN1B* gene were found in patients with multiple endocrine neoplasia syndrome, confirming a tumor suppressor role for p27 in human neoplasia (21).

In general, mouse models have demonstrated tumor suppression by p27 increases with gene dosage, the more p27 the more tumor suppression. However, several exceptions have been reported. For example, tumor development in ErbB2 transgenic mice (22) or Nkx3.1 PTEN-deficient mice (23) was paradoxically delayed in a p27-null background but accelerated in p27 heterozygous mice compared with p27 intact mice. In another example, p27 inhibits intestinal tumor development in mice carrying a mutant *Apc* allele, but not *SMAD3*-deficient mice (24). Thus, tumor suppression by p27 is context dependent and varies depending on the primary oncogenic lesion driving tumor development.

Wnt-1 is a secreted glycoprotein that binds and activates the frizzled receptor, triggering a signaling cascade through *Apc* and glycogen synthase kinase 3-beta (25). In the canonical pathway, this leads to stabilization and nuclear accumulation of β -catenin and Tcf-4-dependent gene expression. Transgenic *Wnt-1* female mice develop mammary tumors with myoepithelial, acinar, or glandular phenotypes (26,27) with a median latency of ~6 months. *Wnt-1* breast tumors express both estrogen and P receptor (28) and tumor development is delayed in ovariectomized or ER- α -deficient *Wnt-1* mice (29), indicating a role for endocrine hormones in this model. The *Apc* tumor suppressor is a key component of the Wnt-signaling pathway and we showed previously that p27 is a potent inhibitor of intestinal neoplasia in *Apc*^{Min} mice (24). Given this background, we tested the hypothesis that p27 suppresses *Wnt-1*-driven breast cancer.

Materials and methods

Mice

B6SJL-Tg(Wnt1)1Hev/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). The *Wnt-1* transgene was backcrossed at least five times to the C57BL/6 strain. These mice were then intercrossed to 129 p27^{+/-} mice (14) to generate C57BL/6 129 (B6129) *Wnt-1* p27-experimental mice. Genotyping protocols are available on request. A second cohort of B6129 *Wnt-1* mice underwent ovariectomy (OVX) at 5 weeks of age and implanted subcutaneous between the scapula with a 90 day release pellet of E (0.1 mg) and P (10 mg) (Innovative Research of America, Sarasota, FL). Pellets were replaced every 90 days. Both male and female mice were observed regularly and killed when tumors reached 1.5 cm in diameter or when they showed symptoms of tumor development at other sites. Mice were necropsied and tumors were removed and sections were frozen in liquid nitrogen or fixed in 10% neutral-buffered formalin and then processed and embedded in paraffin. Hematoxylin and eosin-stained slides from tumors were analyzed for morphologic features as described (30).

Immunohistochemistry and immunoblotting

Immunohistochemical staining for p27 and β -catenin and western blotting for p27 and cyclin D1 from tissue lysates was performed as described previously (24).

Abbreviations: Cdk, cyclin-dependent kinase; E, estradiol; ER, estrogen receptor; OVX, ovariectomy; p27, p27^{Kip1}; P, progesterone.

Serum hormone levels

Blood was collected by cardiac puncture from female mice of all three *p27* genotypes between 15 and 25 weeks of age. Serum was separated from the whole blood clot and frozen at -20°C . Enzyme-linked immunosorbent assay kits (Diagnostics Systems Laboratories, Webster, TX) were used to measure serum E and P levels. Each sample was run in duplicate from 12 mice of each *p27* genotype. A logarithmic curve of absorbance versus concentration was established using the kit standards and GraphPad Prism 3.0; from this curve, the sample concentrations were calculated.

Statistical analysis

Comparisons of breast tumor incidence rates were performed using Cox regression. The development of other tumor types was treated as a competing risk in these analyses. Because of this competing risk, the incidence curves for breast cancer (Figure 1) may not necessarily reflect the underlying incidence rates (Table I). This is particularly notable in Figure 1C, where the apparent incidence of breast tumors in *p27*^{-/-} mice is reduced because of the high incidence of other tumor types, not because of intrinsically lower incidence among mice at risk.

Results

Breast cancer rate in *Wnt-1*-transgenic mice is dependent on gender but not *p27* status

We crossed *p27*-deficient mice to *Wnt-1*-transgenic mice and examined tumor latency in *Wnt-1* mice of all three *p27* genotypes (+/+, +/- and -/-). We examined both males and females to determine if tumor suppression by *p27* interacted with gender. The rate of breast cancer development, expressed as events per 100 weeks, in *p27* intact *Wnt-1* females (2.9) was significantly greater than *Wnt-1* males (0.9) (Figure 1 and Table I). In total, 100% of female mice developed breast cancer by 70 weeks compared with only 60% of male mice by 100 weeks. This shows the prominent influence of sex hormonal environment on *Wnt-1*-driven breast cancer, but also that breast tumors do develop in male mice at fairly high incidence in the absence of high levels of E and P. Loss of either one or both alleles of *p27* had negligible affect on breast tumor development in both male and female mice. The rate of breast cancer in *Wnt-1* female *p27*^{+/+}, *p27*^{+/-} and *p27*^{-/-} mice was similar (2.9, 3.5 and 3.3 events per 100 weeks, respectively). Likewise, the rate of breast cancer in *Wnt-1* male *p27*^{+/+}, *p27*^{+/-} and *p27*^{-/-} mice was similar (0.9, 0.9 and 1.0 events per 100 weeks, respectively) (Figure 1 and Table I). In contrast, *p27*-null mice of both genders developed a greater number of pituitary tumors (six tumors/43 mice) compared with *p27* wild-type mice (zero tumors/41 mice), confirming previous reports of increased susceptibility of *p27*-deficient mice to these tumors (12–14). We conclude that the difference in breast tumor development that exists between male and female mice is largely independent of *p27* status.

Tumor morphology and grade

To determine if *p27* status affected tumor grade, breast tumors from each genotype were evaluated histologically and categorized by morphological subtypes (acinar, papillary, cribriform, comedo or solid), and for tumor grade, which encompasses degree of tubularity, nuclear pleomorphism, mitotic and apoptotic activity (30). Morphology of *Wnt-1* breast tumors was consistent with published studies (27) and no differences were observed between *p27* genotypes with respect to morphological subtype or tumor grade (data not shown).

β -Catenin staining in *Wnt-1* breast tumors

The lack of cooperation between *p27* deficiency and *Wnt-1* in breast tumor development differed from the previously reported strong cooperation between *p27* loss and *Apc* mutation in gastrointestinal neoplasia (24). Both *Wnt-1* and mutant *Apc* are thought to act through a similar signaling pathway, culminating in stabilization of nuclear β -catenin and Tcf-dependent gene expression (25). However, direct comparison of nuclear β -catenin revealed significant differences. Prominent nuclear β -catenin was clearly evident in the majority of cells from *Apc*^{Min} intestinal adenomas, whereas breast tumors from *Wnt-1* mice were uniformly negative for nuclear β -catenin staining

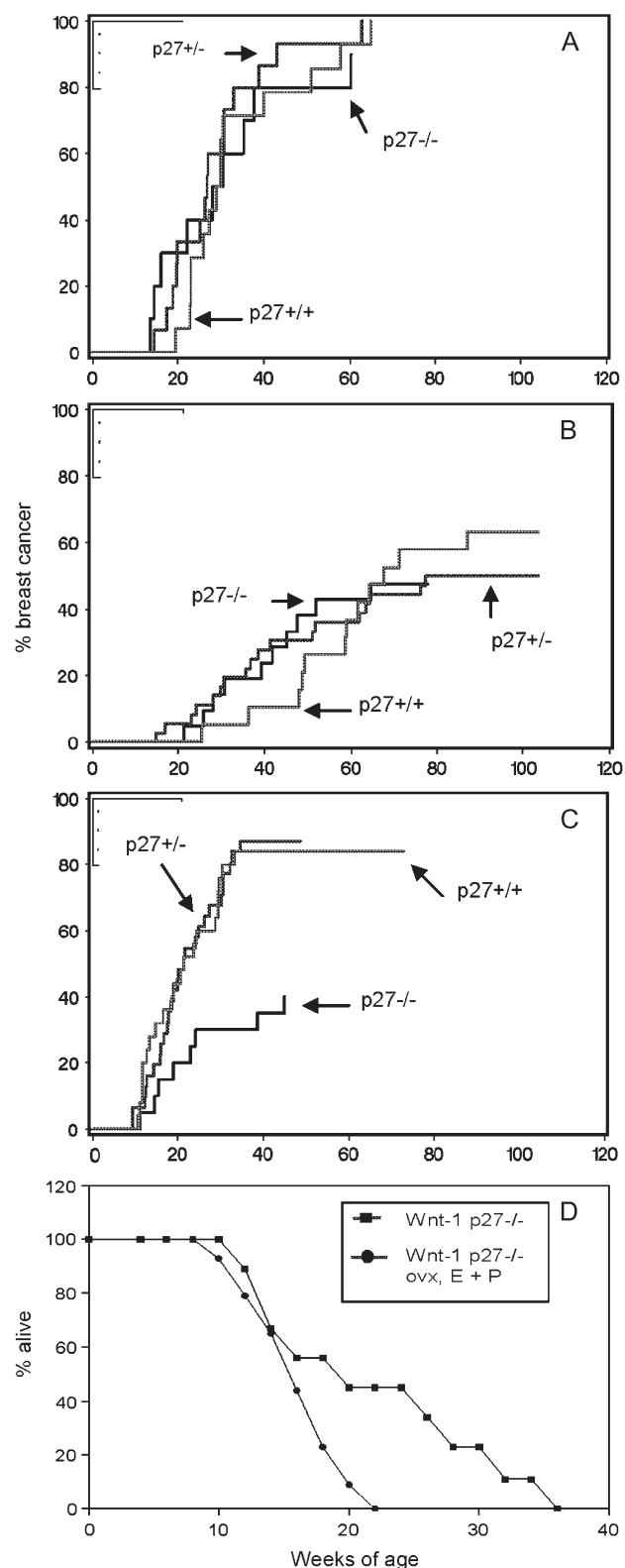


Fig. 1. Effect of *p27* genotype on breast tumor latency. The percentage of mice that developed breast cancer is plotted versus age in weeks. (A) Intact B6129 *Wnt-1* female mice of the indicated *p27* genotypes. (B) Intact B6129 *Wnt-1* male mice of the indicated *p27* genotypes. (C) Ovariectomized, estrogen and P-supplemented B6129 *Wnt-1* female mice of the indicated *p27* genotypes. (D) Kaplan-Meier survival analysis of *Wnt-1 p27*^{-/-} mice versus *Wnt-1 p27*^{-/-} ovariectomized and implanted with E + P.

Table I. Statistical analysis of tumor development in B6129 *Wnt-1* mice

Sex	Genotype	<i>n</i>	Breast cancer rate ^a	<i>P</i> -value ^{b,c}	Other cause rate ^a	<i>P</i> -value ^b
Intact female	<i>p27</i> ^{-/-}	10	3.3	0.68	0.4	
	<i>p27</i> ^{+/-}	15	3.5	0.97	0.0	
	<i>p27</i> ^{+/+}	14	2.9	0.48, 0.44	0.0	0.20
Male	<i>p27</i> ^{-/-}	21	1.0	0.82	1.1	
	<i>p27</i> ^{+/-}	36	0.9	0.56	0.9	
	<i>p27</i> ^{+/+}	19	0.9	0.58, 0.98	0.6	0.0005
OVX female	<i>p27</i> ^{-/-}	20	2.0	0.44	3.0	
	<i>p27</i> ^{+/-}	31	3.8	0.27	0.6	
	<i>p27</i> ^{+/+}	25	3.4	0.25, 0.93	0.6	0.0001

OVX: ovariectomized and implanted with pellets of estrogen and P.

^aEvents per 100 weeks of observation.

^bFrom Cox regression.

^cThe first line *P*-value is the overall test of equality. The second line is the test of *p27*^{+/-} versus *p27*^{-/-}. The third line lists the tests of *p27*^{+/+} versus *p27*^{-/-} and *p27*^{+/+} versus *p27*^{+/-}.

(Figure 2B). To determine if this differential activation of β -catenin was due to tumor type or to differential signaling from *Wnt-1* versus mutant *Apc*, we took advantage of the fact that *Apc*^{Min} mice from our colony (24) occasionally developed breast tumors. Breast tumors from *Apc*^{Min} mice displayed small, focal areas of nuclear β -catenin staining, surrounded by a majority of unstained tumor cells, as compared with uniformly positive nuclear staining in *Apc*^{Min} intestinal tumors (Figure 2B). This shows that breast tumors driven by mutant *Apc* exhibit reduced activation of β -catenin compared with intestinal tumors driven by the same mutation, indicating tumor-specific regulation of β -catenin. Thus, differential activation of β -catenin between breast and intestinal tumors provides at least one explanation for the dissimilar tumor suppressing effects of *p27* in the two models.

p27 expression in *Wnt-1* breast tumors

p27 protein levels are frequently reduced in tumors due to increased *p27* degradation, reduced transcription or other pathways (2). We examined *p27* expression in *Wnt-1* breast tumors with both immunohistochemistry and western blot analysis. Prominent nuclear staining for *p27* was seen in normal breast ductal epithelia (Figure 2A). *Wnt-1* breast tumors showed more variable *p27* expression, but prominent nuclear staining was clearly detected in many cells throughout the tumors. Immunoblotting of nuclear/cytoplasmic fractions from tissue lysates showed variable *p27* expression in breast tumors, but an overall increase compared with normal breast tissue (Figure 2C). No major difference was seen in *p27* levels in tumors from male or female mice. *p27* was markedly reduced in *Apc*^{Min} intestinal adenomas relative to normal intestinal tissue (24), further indication of differential signaling pathways through *p27* in *Wnt-1* breast tumors compared with mutant *Apc* intestinal tumors. Cyclin D1 levels were markedly increased in *Wnt-1* breast tumors consistent with increased proliferation compared with normal breast tissue (31).

p27 deficiency affects circulating levels of E and P

Previous studies revealed that *Wnt-1* breast tumors express ER- α (28) and that breast tumor development is delayed in ER- α -null or -ovariectomized mice (29,32), indicating a supporting role for ovarian-derived hormones such as E and P in *Wnt-1*-driven tumor development. *p27*-null female mice are infertile, exhibiting a disordered estrus cycle and impaired formation of the corpus luteum (12–14). As E and P are produced by the corpus luteum and both play important roles in the natural history of breast cancer, we asked if E and P levels were altered in *p27*-null mice. The mean serum P concentration in *p27*^{-/-} mice (13.9 ng/ml) was significantly lower than wild-type (24.3 ng/ml) ($P < 0.02$) or *p27*^{+/-} (29.3 ng/ml) ($P < 0.01$) mice (Figure 3). Serum P concentrations did not differ significantly between *p27*

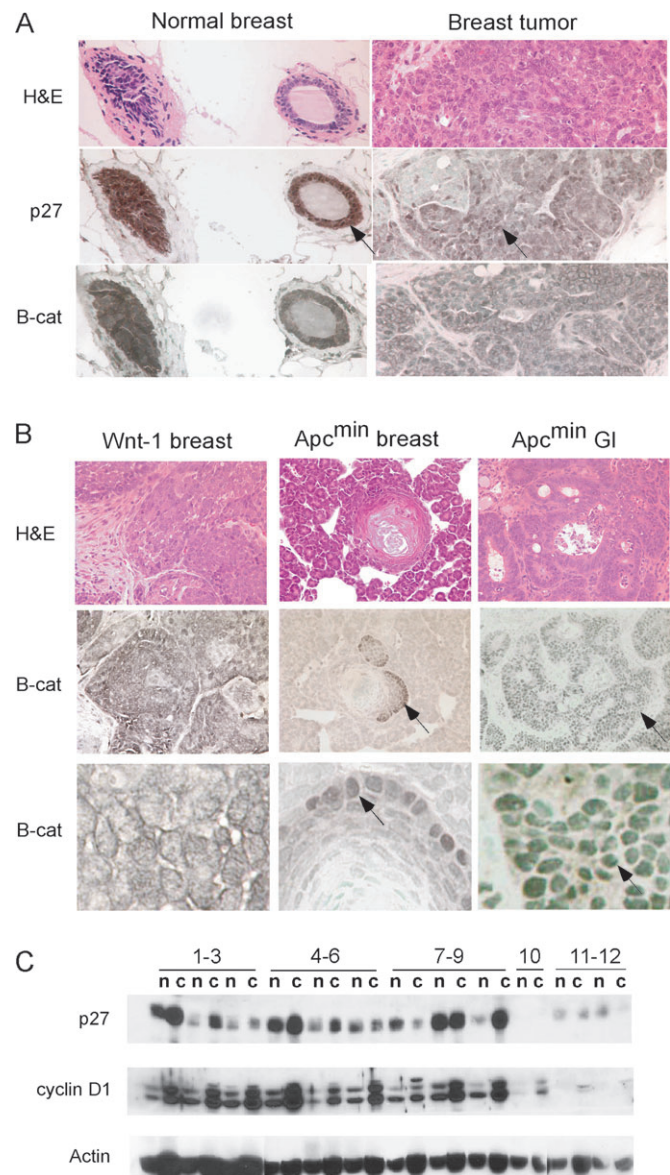


Fig. 2. *p27* and β -catenin (B-cat) expression in *Wnt-1* tumors. (A) *p27* and B-cat staining of normal breast and breast tumor from *Wnt-1* mice. Arrows point to positive cells. Left column photographs taken at $\times 100$ and right column at $\times 600$ magnification. (B) Comparison of B-cat staining in breast and intestinal (gastrointestinal) tumors from *Apc*^{Min} mice and *Wnt-1* transgenic mice. Middle row photographs taken at $\times 100$ and bottom row at $\times 600$ magnification. (C) Immunoblot analysis of *p27* and cyclin D1 in *Wnt-1* breast tumors from the following mice. Lanes 1–3: intact female; lanes 4–6: E- and P-treated female; lanes 7–9: male; lane 10: *p27* null; lane 11 and 12: normal breast. n, nuclear fraction and c, cytoplasmic fraction.

wild-type and *p27*^{+/-} mice ($P > 0.28$). The mean serum E concentration did not differ appreciably between wild-type (92.4 pg/ml) and *p27*^{-/-} (104.6 pg/ml) female mice; however, there was greater intermouse variation in *p27*^{-/-} mice, with values ranging from 34.0 to 188.0 pg/ml (Figure 3). E levels in *p27*^{+/-} mice (73.6 pg/ml) were significantly reduced compared with *p27*^{-/-} mice ($P < 0.04$ using a two-sided *t*-test). This suggests E levels are less tightly regulated in *p27*-deficient mice, consistent with previous findings of perturbed estrous cycling (13).

Circulating levels of E and P affect tumor predisposition of *p27*-null mice

Having shown that *p27*-deficient mice have altered the serum levels of E and P, we asked if this affected their susceptibility to breast or other

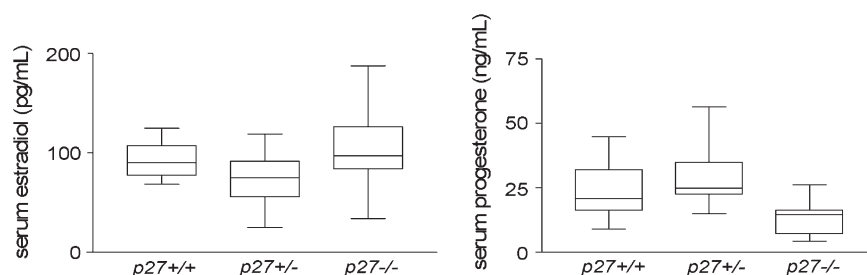


Fig. 3. Serum concentrations of E and P in p27-deficient mice. Median concentrations are plotted with 25–75 percentiles (boxes) and ranges (vertical bars).

hormonally responsive cancers. To address this, we equalized E and P levels between p27 genotypes and measured tumor susceptibility. A cohort of *Wnt-1* female mice of all three p27 genotypes was subjected to OVX and implanted with slow release pellets of E and P (see Materials and Methods). Compared with intact mice, the rate of breast tumor development in E- and P-implanted mice was modestly increased in p27 wild-type and p27 heterozygous mice (from 2.9 to 3.4 and 3.5 to 3.8 events per 100 weeks, respectively) but appeared to be decreased in p27-null mice (from 3.3 to 2.0 events per 100 weeks) although this did not reach statistical significance (Figure 1, Table I). Measurement of breast cancer rate in p27 nulls was complicated by early lethality from other tumor types, notably pituitary, stomach and duodenal tumors (Figure 1D). The rate of development of these non-breast tumors in treated p27^{-/-} mice was independent of *Wnt-1* (data not shown), was significantly greater than in treated p27^{+/-} or p27^{+/+} mice (3.0, 0.6 and 0.6 events per 100 weeks, respectively, $P = 0.0001$) and was increased compared with intact untreated p27^{-/-} mice (0.4 events per 100 weeks, $P = 0.001$) (Table I). Thus, OVX followed by E and P pellets only affected tumor development in p27^{-/-} mice, indicating that the hormonal dysfunction of p27-null mice influences their tumor predisposition. Ninety-three percent (13/14) of E- and P-treated p27-null mice developed pituitary tumors of both the distal and intermediate lobes, compared with only 12% (2/16) in E- and P-treated wild-type and 15% (2/13) in intact p27-null females over a similar time frame ($P < 0.001$, Chi squared test). In addition, 71% of E- and P-treated p27-null mice developed stomach lesions, located within the cardiac region of the glandular stomach or at the duodenal junction, with an average latency of 4.0 ± 0.8 months. These lesions were often large and in several cases perforated through the stomach wall or obstructed the duodenal passage (Figure 4). Histologically, these were classified as a mixture of hyperplasias, adenomatous polyps and cystic adenocarcinomas of the gastric stomach as well as adenomas of the duodenum. Sixty percentage of intact p27^{-/-} mice also developed stomach tumors, but with a longer latency of 5.5 ± 1.7 months, whereas none of the p27^{+/-} or p27^{+/+} mice developed such tumors. E- and P-treated mice of all genotypes also presented with uterine endometrial hyperplasia, consistent with known effects of sustained exposure to E and P. Together, these results show that (i) p27 does not significantly alter *Wnt-1*-driven breast cancer in either male or female hormonal milieu. (ii) p27-null mice are susceptible to gastric cancer. (iii) Tumor spectrum and especially tumor kinetics of p27-null mice is influenced by endocrine dysfunction and when rendered hormonally equivalent, p27-null mice exhibit an even greater susceptibility to tumor development.

Discussion

Hormones play a major role in breast cancer in humans (5). In *Wnt-1* mice, breast cancer was markedly delayed in males relative to females, indicating the strong hormonal dependence of this tumor model. Germline p27 deficiency did not significantly alter the latency of breast tumors in either male or female mice, indicating that the effect of the sex hormonal environment on breast cancer development is largely p27 independent. Further, the histological subtypes and aggressiveness of breast tumors from p27-null mice were indistin-

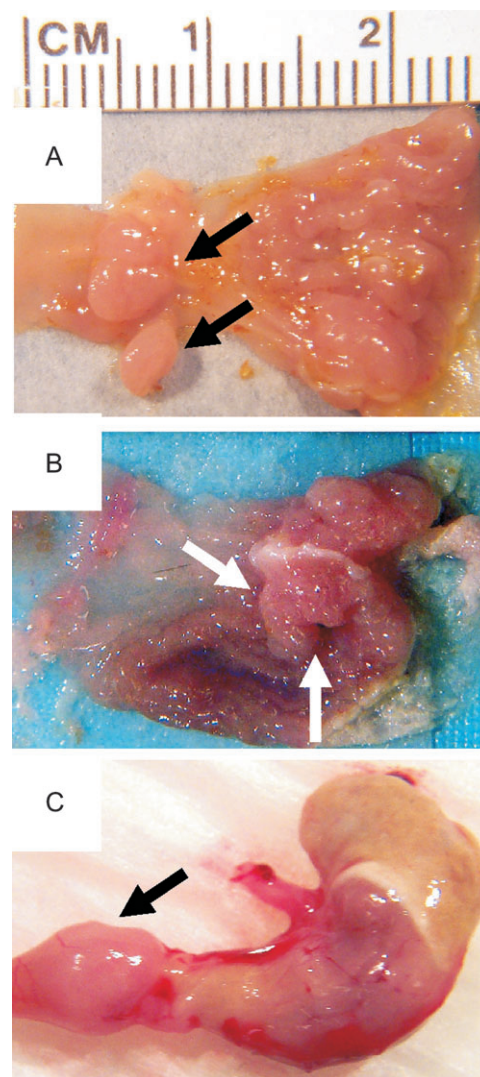


Fig. 4. Gastric tumors from p27-null mice. Arrows point to lesions at gastric region of the stomach or at the duodenal junction.

guishable from wild-types. These results, together with the retention of p27 expression in *Wnt-1* breast tumors, indicate that the initiation and growth of these tumors is not inhibited by p27. Jackson *et al.* (33) showed that germline p27 deficiency did not modify breast tumor latency in a Ras transgenic model. These results showing little or no affect of p27 are different to that seen in an ErbB2 transgenic model, where p27 heterozygosity accelerated and nullizygosity delayed breast cancer (22). The delayed tumorigenesis in the absence of p27 was attributed to a requirement for p27 to assemble functional

cyclin D–Cdk complex and drive cell proliferation. Consistent with this idea, breast cancer in ErbB2-transgenic mice was completely eliminated in cyclin D1-null mice, indicating a strict requirement for cyclin D1 (34). Together, these findings reinforce the idea that tumor suppression by p27 is pathway specific (24) and suggest that the role of p27 in human cancer is likely strongly influenced by the primary underlying genetic lesion within each tumor type.

The lack of cooperation between p27 loss and *Wnt-1* is also in contrast with potent tumor suppression by p27 seen in mutant *Apc*-driven intestinal tumors (24). This differential effect of p27 between the two models could be attributed to differential signaling between transgenic *Wnt-1* in breast tumors and mutant *Apc^{Min}* in intestinal tumors. *Wnt-1* was shown to expand the mammary stem cell pool and this is a proposed explanation for heterogeneous cell types seen in *Wnt-1* tumors (35,36). Further, nuclear β -catenin staining was much more prominent in *Apc^{Min}* intestinal tumors compared with breast tumors from either *Wnt-1* or *Apc^{Min}* mice, indicating differential activation of β -catenin in breast compared with intestinal tumors. The increase in cyclin D1 seen in *Wnt-1* breast tumors could be due to non-canonical Wnt signaling. Interestingly, Yu *et al.* (34) showed that cyclin D1 deletion had only a modest effect on breast tumor latency in *Wnt-1* transgenic mice, which together with our results indicate that *Wnt-1*-driven tumor formation is largely independent of the cyclin D1/p27 axis.

In addition to the known function of p27 as a cell autonomous tumor suppressor (37), these studies identify a non-cell autonomous endocrine-mediated pathway that modifies the tumor predisposition of p27 deficient mice. p27-null female mice exhibit altered endocrine function, as shown by reduced fertility, aberrant corpus luteum formation and altered estrus cycling (13). Tong *et al.* (38) reported that p27 is required for the proper coupling of differentiation and growth arrest during the granulosa to luteal transition. As the corpus luteum produces E and P, this provides an explanation for the aberrant levels of serum P and E seen in p27 deficient mice. When rendered hormonally equivalent to wild-type mice by OVX and E and P implants, p27-null mice developed adenomas and adenocarcinomas of the stomach and duodenum, as well as accelerated distal and intermediate lobe pituitary tumors. Ikeda *et al.* (39) previously reported accelerated pituitary tumorigenesis in E-treated p27-deficient mice. These results indicate a component of the susceptibility of p27-deficient mice is non-cell autonomous and attributable to an altered hormonal environment. The predisposition of p27-null mice to gastric tumors was also seen in p27-null mice after infection with *Helicobacter pylori* (40). In human gastric cancer, reduced p27 protein expression correlates with advanced stage, invasiveness and prognosis (41–45) and a protective effect of estrogen in stomach cancer has been reported (46). These findings demonstrate a causal role for p27 in suppression of gastric cancer. Further studies will be required to address the mechanisms of tumor suppression by p27 in hormonally dependent cancers.

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