p27 as a target for cancer therapeutics

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Introduction

p27kip1 is a member of the cdk inhibitory proteins, with an important role coordinating the activation of cyclin E-cdk2 with accumulation of cyclin D-cdk4 and initiating the timely exit of cells from the cell cycle in response to antimitogenic signals. Three features of p27 make it a strong candidate as a target to consider in cancer therapy. First, while not mutated or lost at the gene level, the abundance of the protein has proven to be a reliable prognostic marker for disease progression in a diverse group of human neoplasms. This has been recapitulated in a number of organ-specific mouse models. Second, many cancer therapies are effective only in cells that are actively engaged in the cell cycle. Given that p27 inhibits cell proliferation, its expression may affect the efficacy of the therapies. Third, the amount of p27 in a cell is largely controlled posttranscriptionally by a number of pathways implicated in human cancer. These features are interwoven such that modulating p27 levels can have unforeseen consequences on therapy. In a clinical setting, growth arrest is a double-edged sword that might inadvertently lead to increased cell survival and resistance to cytotoxic agents, while increasing proliferation may increase chemotherapeutic efficiency.

p27 and cell cycle control

The "Rb pathway" is the major route by which mitogenic signals promote proliferation (Sherr and McCormick, 2002). Disruption of this pathway, by either overexpression of cyclin D, loss of the Ink4 inhibitor p16, mutation of cdk4 to a p16-resistant form, or even loss or mutation of Rb itself, are commonly associated with increased proliferation of cancer cells. An alternate route to control proliferation is through antimitogenic signals, which lead to cell cycle exit. This route functions through the cdk inhibitors. $p27^{KIP1}$ (hereafter referred to as p27) is one member of the Cip/Kip family of cdk inhibitors, whose principle role in cell cycle exit is to inhibit the cyclin E-cdk2 kinase. In proliferating cells, p27 is primarily associated with cyclin D-cdk4/6 complexes, but these complexes are catalytically active. The sequestration of p27 by cyclin D-cdk4/6 complexes effectively frees cdk2 from inhibition and allows both cdk4/6 and cdk2 to remain active. The movement of p27 helps to establish a balance between proliferation (when associated with cdk4) and arrest (when associated with cdk2).

The amount of p27 increases in quiescent cells or cells undergoing differentiation, and it is a rate-determining component of cell cycle exit in a number of cell types. Although it is unclear how differentiation signals trigger accumulation of p27, it is clear that mitogens and extracellular matrix adhesion signals can promote p27 degradation, allowing quiescent cells to reenter the cell cycle. p27 knockout animals develop hyperplasia in multiple organs, resulting in a mouse roughly 20%–30% larger than their wild-type counterparts, consistent with a role for p27 in both differentiation and proliferation (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Surprisingly, p27 null animals are relatively free of malignancy, with the exception of pituitary hyperplasia that can develop into adenoma with age and prostatic hyperplasia that becomes increasingly severe with age (Cordon-Cardo et al., 1998). Thus, the absence of p27 alone indicates that it might be a weak tumor suppressor of a narrowly defined group of cells, largely affecting the decision of cells to exit the cell cycle. However, its absence alone cannot provide the proliferation impetus required for most neoplasms.

p27 and cancer

p27 does not follow Knudson's classic "two-hit hypothesis" of tumor suppression; homozygous loss or silencing of the locus in human tumors is extremely rare. However, an inverse correlation between p27 protein levels and prognosis was first noted in colon cancer and later in cancers of the breast, prostate, bladder, lung, glia, liver, larynx, ovary, stomach, and other tissues (reviewed extensively in Lloyd et al., 1999; Philipp-Staheli et al., 2001; Slingerland and Pagano, 2000; Tsihlias et al., 1999). The loss of p27 function in human cancers and the prognostic significance associated with reduction in p27 suggested that it might be a valuable target for both stratifying patient risk and, perhaps, selecting treatment.

Nevertheless, before considering p27 in this way, one first needed to ascertain whether loss of p27 was a causal or consequential effect. This was addressed in a number of mouse models where the effect of p27 deficiency on tumor progression was analyzed. In these models, p27 was shown to be a dosage-dependent tumor suppressor. Carcinogen challenge demonstrated that the loss of a single allele of p27 increased the frequency and decreased the latency of tumors to a level intermediate to that seen in wild-type and null counterparts (Fero et al., 1998). Furthermore, the remaining allele was intact in these tumors, leading many to call p27 a haploinsufficient tumor suppressor. However, haploinsufficiency is when the phenotype of the null is equivalent to the heterozygote and animals nullizygous for p27 have an even further increased rate of tumor formation in the presence of carcinogens. Consequently, dosage-dependent is probably more appropriate. A similar dosage effect may be at work in human tumors, where, in some leukemias and lymphomas, hemizygous deletion of the p27 locus 12p13 has been detected without additional mutation of the second allele (Philipp-Staheli et al., 2001).

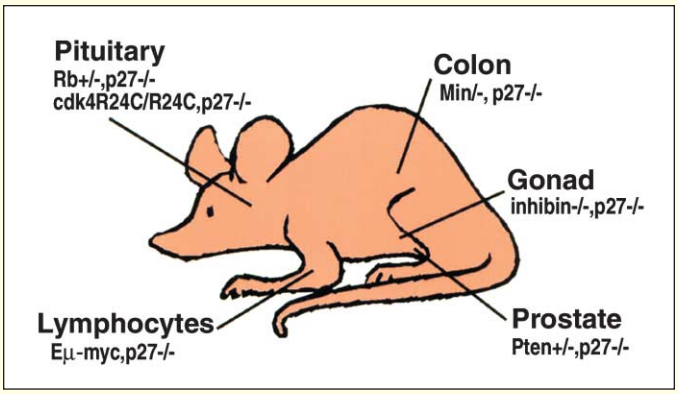

Figure 1. Mouse models recapitulate the role of p27 in human cancers

While p27 null mice are relatively tumor-free, p27 loss enhances the malignancy and frequency of tumor formation in cooperation with different oncogenic stimuli. p27 null animals were crossed with *Rb*$^{+/-}$, cdk4R24C/R24C, *Min*$^{/-}$, Eμ-*myc*, *inhibin*$^{-/-}$, or *Pten*$^{+/-}$ animals to generate compound phenotypes. Refer to the text for the specific references to the studies that generated this figure.

p27 deficiency also enhanced the rate of tumor formation in the mouse in a number of tissues in cooperation with a number of specific oncogenic stimuli (Figure 1). In the prostate, about half of the *Pten*$^{+/-}$ mice develop prostatic intraepithelial neoplastic (PIN) lesions, with none progressing to carcinoma-in-situ. When *Pten*$^{+/-}$ mice are crossed into a p27$^{-/-}$ background, the incidence of PIN lesions increases to 100% and many develop to carcinoma-in-situ, with an occasional invasive tumor observed (Di Cristofano et al., 2001). Thus, p27 loss not only increased the number of animals afflicted, it also accelerated the rate at which tumors progressed. p27 appears to be dosage-dependent in this context: the frequency and latency of tumors in the *Pten*$^{+/-}$; *p27*$^{+/-}$ animals are intermediate to those seen in the *Pten*$^{+/-}$ and the *Pten*$^{+/-}$; *p27*$^{-/-}$ animals. The enhancing effect of p27 deficiency is not restricted to a particular oncogenic event or even a particular tissue. Similar observations have been reported in the pituitary of both *Rb*$^{+/-}$; *p27*$^{-/-}$ (Park et al., 1999) and cdk4R24C/R24C; *p27*$^{-/-}$ mice (R24C is a knockin allele of an Ink4 resistant cdk4 mutant) (Sotillo et al., 2001), in the gonads using *inhibin*$^{-/-}$; *p27*$^{-/-}$ (Cipriano et al., 2001), in the colon using *Min*$^{/-}$; *p27*$^{-/-}$ (Philipp-Staheli et al., 2002), and more recently, in lymphocytes using Eμ-*myc*; *p27*$^{-/-}$ (Marins and Berns, 2002). In the Eμ-*myc* model, loss of p27, but not loss of p21, synergizes with myc expression (Marins and Berns, 2002), highlighting an important distinction between these two inhibitors. The fact that p27 loss in mice significantly affects tumor progression, in conjunction with the observations that its levels are reduced in human tumors, suggests that restoring p27 might have a beneficial clinical effect. On the other hand, inhibiting proliferation may contribute to treatment resistance to current therapies, because many of these are dependent on cells being engaged in the cell cycle. Thus, it is important to determine how low p27 status contributes to the evolution of the tumor and modulates the responses of that tumor type to preexisting chemotherapies.

It is well established that p27 is an important component of the pathways by which cells respond to antimitogenic signals, and these signals can interfere with tumor development in two ways (Massagué et al., 2000; Sherr, 2000). First, because antimitogens obviously inhibit cell proliferation, the loss of p27 might collaborate with an oncogenic event to increase the rate of cell proliferation. There is ample evidence in lymphoma, bladder, prostate, and liver cancer (Slingerland and Pagano, 2000) that p27 loss is correlated with an increase in proliferation index. This is also exemplified in the *Pten*/*p27* and *Min*/*p27* mouse models. In *Pten*$^{+/-}$; *p27*$^{-/-}$ animals, the uninvolved prostate glands show increased Ki67 staining without any change in the rate of apoptosis (Di Cristofano et al., 2001).

However, low p27 expression does not correlate with proliferation index in breast, colon, esophagus, thyroid, and astrocytic cancers (Lloyd et al., 1999; Tsihlias et al., 1999). This raised the possibility of another distinct mechanism by which reducing p27 levels could contribute to the neoplastic state. Oncogenic events sensitize cells toward an apoptotic end when they are confronted with antiproliferative signals that interfere with the replication of their DNA (de Stanchina et al., 1998). Because antimitogens can also prevent DNA replication, they can also trigger apoptosis in oncogenically activated cells. Thus, the loss of p27 may desensitize tumor cells to antimitogenic signals, preventing apoptosis during their evolution. This principle is exemplified in the *Rb*/*p27* model. In *Rb*$^{+/-}$; *p27*$^{-/-}$ animals, p27 deficiency enhances the rate and aggressiveness of pituitary tumors, while the proliferative index of the tumors is the same as in *Rb*$^{+/-}$ animals (Park et al., 1999). Recent genetic evidence suggests that the absence of p27 prevented *Rb*$^{-/-}$ cells from undergoing apoptosis when exposed to normal levels of the antiproliferative cytokine dopamine, a signal that promotes their withdrawl from the cell cycle (Carneiro et al., 2003).

The evidence that reduced p27 may contribute to tumor development by either increasing the proliferation of cells (Pten prostate model) or decreasing their apoptosis (Rb pituitary model) might explain why the loss of p27 is a common marker among many different tumor types. The loss of p27 attenuates the cell-type specific response to its antimitogenic environment.

The clinical promise and problem with p27

The prognostic and treatment-predictive significance of p27 status cannot be understated. Patients with tumors that have low p27 levels have an inferior prognosis relative to those with high expression. Decreased overall or disease-free survival has been seen in numerous human cancers, and suggests that the clinician should treat these patients aggressively. One approach considered is gene therapy to restore p27, either directly by using adenoviral vectors (Katner et al., 2002) or indirectly by chemically modulating signaling pathways that affect its localization (Shin et al., 2002). Both of these approaches have seen validation as cytostatic agents in cell lines and xenograft models, although no human trials have been reported. Enforced expression of p27 can also have apoptotic consequences as reported in a transduced oral cancer cell line (Supriatno et al., 2002) and in T cell acute lymphoblastic leukemia cells (Barata et al., 2001).

On the other hand, accumulating p27 might have negative consequences in treatment outcome. High levels of p27 might inhibit the efficiency of chemotherapies that rely on interfering with proliferation to trigger a cytostatic or cytotoxic response through checkpoint activation (reviewed in Chauffert et al., 1999). In fresh breast cancer specimens analyzed directly for p27 status and chemotherapeutic sensitivity by histoculture drug response assay, a marked association between increased

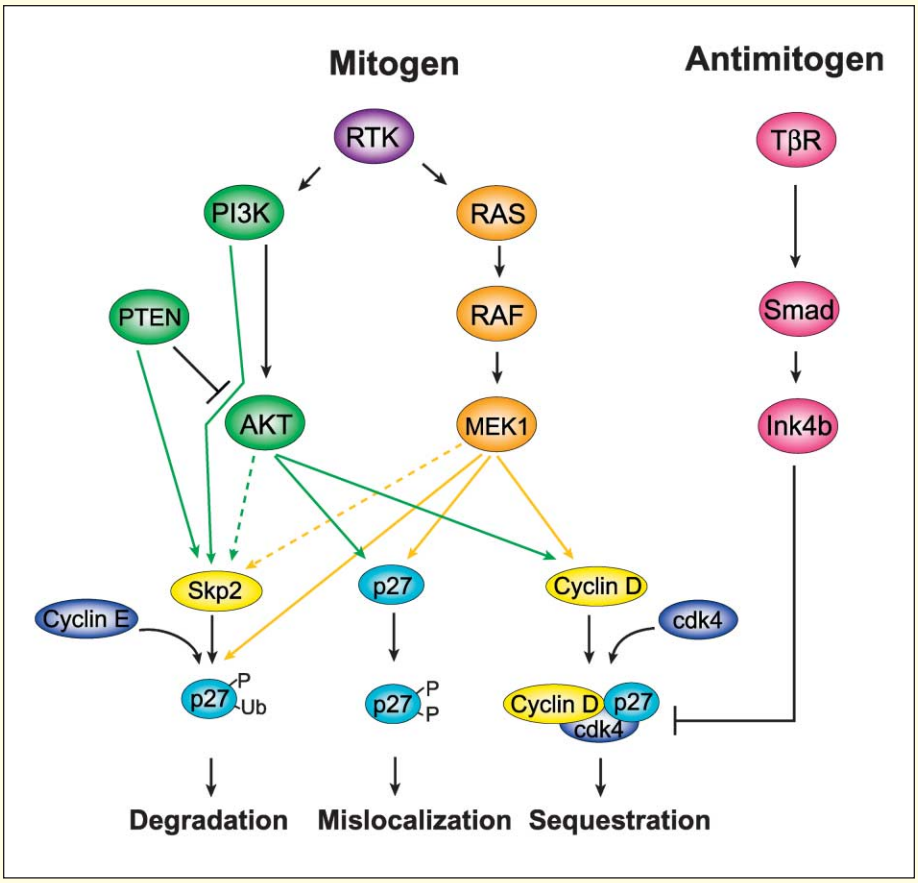

Figure 2. p27 is targeted by multiple oncogenic stimuli

The PI3K/Pten/Akt pathway (1) induces Skp2, resulting in increased ubiquitination and the subsequent degradation of p27, (2) stabilizes cyclin D1 via inactivation of GSK3-β, resulting in increased cyclin D1 sequestration of p27, and (3) directly phosphorylates p27, causing mislocalization to the cytoplasm. The Ras/Raf/Mek1 pathway also (1) increases p27 degradation, (2) induces cyclin D1 transcription, and (3) causes p27 mislocalization to the cytoplasm, perhaps via interactions with Jab1 or KIS phosphorylation. All of these events result in the inactivation of p27's inhibitory activity. Increased cyclin E may cause increased p27 phosphorylation and subsequent degradation. While Pten and PI3K have been shown to regulate Skp2 levels, a direct link between Akt and Skp2 has not been established, and Akt-independent functions of Pten and PI3K have been described. The MAPK pathway has been shown to regulate p27 stability via phosphorylation and degradation, but a direct link between MAPK and Skp2 has not been demonstrated. A direct correlation between cyclin E, Jab1, KIS, and p27 in human tumors has not been demonstrated. Loss of p27 would attenuate antimitogenic signaling, rendering a cell resistant to certain growth-arresting stimuli, such as TGF-β.

p27 staining and resistance to doxorubicin was observed (Yang et al., 2000). Antisense techniques applied in tissue culture increased chemosensitivity, suggesting promise for such an approach in human tumors as well (Achenbach et al., 2000; St Croix et al., 1996).

The duality of p27 as a prognostic marker and the fact that p27 status can affect cell proliferation should be used to inform the treatment of a patient. Low p27 status informs the clinician that he or she is facing an aggressive tumor, and also predicts that it might respond well to proliferation inhibitory chemotherapies, whereas high p27 status informs the clinician that he or she is facing an indolent tumor which will only poorly respond to such interventions. Thus, knowing the status of p27 in a particular tumor should become *de rigueur* in the clinic when choosing a particular therapy.

Modulating p27 levels

One important clinical question is how one might modulate the level of p27 in order to enhance other therapies. In human tumors, p27 itself or its activity appear to be lost by increased degradation, cytoplasmic mislocalization, or sequestration. The abundance of p27 is controlled in a cell-type-specific and signal-specific manner by the integration of mitogenic and antimitogenic signaling pathways that affect its translation, stability, and localization. The Ras/Raf/Mek1 and PI3K/Akt pathways, commonly mutated in human cancer, can impact directly on the abundance and activity of p27, suggesting that targeting these pathways may indirectly affect the activity of p27 and the response of cells to other chemotherapeutic treatments.

p27 mRNA levels remain constant throughout the cell cycle, while its protein levels are controlled by ubiquitin-mediated proteolysis at the G0–G1 and G1–S borders (Nakayama et al., 2001). Not surprisingly, the abundance of p27 in many tumors correlates with ubiquitin-dependent proteolytic activity. Recently, a direct inverse correlation between reduced p27 levels and increased Skp2 levels, a key component of the p27 ubiquitin-ligase, has been seen in colorectal tumors, oral squamous cell carcinomas, and lymphomas (Chiarle et al., 2002; Gstaiger et al., 2001; Hershko et al., 2001; Kudo et al., 2001; Latres et al., 2001). In theory, many additional oncogenic insults might directly impinge on p27 stability. For example, overexpression of PI3K or loss of *Pten* have been shown to directly increase Skp2 levels (Mamillapalli et al., 2001). Numerous studies have demonstrated that overexpression of Her-2/neu or Ras, and activation of the MAPK pathway reduces p27 stability (Pruitt and Der, 2001). Likewise, overexpression of cyclin E, in breast, stomach or colon carcinomas, might increase p27 phosphorylation and degradation.

p27 activity also appears to be directly targeted by its mislocalization to the cytoplasm in colon, ovarian, breast, and thyroid tumors (Baldassarre et al., 1999; Ciaprrone et al., 1998; Hurteau et al., 2001). Cytoplasmic p27 appears to directly correlate with poor long-term survival and tumor grade in Barrett's associated adenocarcinoma of the esophagus and breast carcinoma (Liang et al., 2002; Shin et al., 2002; Singh et al., 1998; Viglietto et al., 2002). Mislocalization effectively inactivates p27 inhibitory activity, as cytoplasmic p27 is partitioned from its nuclear cyclin-cdk targets. Cytoplasmic mislocalization of p27 may also be the result of a variety of different oncogenic assaults. For example, Ras or Her-2/neu overexpression has been shown to increase p27 levels in the cytoplasm (Liu et al., 2000; Yang et al., 2000), and oncogenically activated Akt was shown to directly phosphorylate a Thr residue (T157) in the p27

NLS motif, causing p27's cytoplasmic mislocalization (Liang et al., 2002; Shin et al., 2002; Viglietto et al., 2002).

Sequestration of p27 in the cytoplasm by cyclin D-cdk4/6 complexes is yet another way that p27 might be inactivated in human tumors. Overexpression of cyclin D3 in thyroid cancers caused p27 cytoplasmic sequestration by cyclin D3-cdk complexes (Baldassarre et al., 1999). Both the Ras/Raf/Mek1 and PI3K/Akt pathways may impinge on p27 localization indirectly by their direct effects on cyclin D levels. MAPK activation upregulates cyclin D1, and AKT-dependent phosphorylation of GSK3-β inhibits cyclin D1 degradation (Pruitt and Der, 2001). Both of these mechanisms would increase the subsequent sequestration of p27, and prevent association with its targets.

Thus, the inactivation of p27 can be achieved by a number of different oncogenic stimuli, including amplification of Ras, cyclin D, loss of Pten, increased RTK activity (HER-2, IGFR, EGF), increased Akt activity, and increased cyclin E or Skp2 (Figure 2). In fact, p27 may be a powerful prognostic marker because it is the readout of multiple different pathways involved in the development of tumors. It also highlights the potential overlap of these pathways. For example, increased Akt activity might increase Skp2 and cyclin D1 levels and directly phosphorylate p27, all of which may collaborate to promote p27 mislocalization and/or degradation. The fact that most of the control of p27 abundance is posttranscriptional suggests that a proteomic survey, in addition to a genomic survey which is limited to transcriptional changes, would have prognostic clinical value. Likewise, attempts to modulate p27 activity or amount, either directly through its expression or indirectly by targeting signaling pathways that control it, may be a promising approach for combinatorial chemotherapy.

Future challenges

Clinically, the efficacy of many chemotherapies relies on their ability to induce DNA damage. p27 induces cell cycle exit, and thus, its expression can interfere with treatments that work better in proliferating cells. In addition, the occasional apoptotic effect of p27 expression is cell type-specific and dependent on contextual clues which are only poorly defined at this time. Thus, we need to be able to predict how cells in a particular tumor will respond to accumulation of p27. Will this response be beneficial clinically? Will it interfere with the efficacy of therapies currently in use, or could it be combined with novel therapeutic approaches?

One might imagine that therapies could be designed which capitalize on our knowledge of p27's role in a specific tumor to target chemotherapeutic resistant cells. If we could treat initially resistant cells with an agent that interferes with p27 accumulation or function, we might be able to "push" those cells back into the cycle, exposing them to the cytotoxic agents when they are most sensitive; i.e., at a time they are proliferating. Repeated transitioning in and out of cycle with selective application of cytotoxic agents might eventually allow all cancer cells to be eliminated. This type of thinking represents the integration of what we have learned about p27 in the laboratory and its possible applications into the clinic.

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