p21(WAF1) Mediates Cell-Cycle Inhibition, Relevant to Cancer Suppression and Therapy

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Abstract

p21 (WAF1/CIP1; CDKN1a) is a universal cell-cycle inhibitor directly controlled by p53 and p53-independent pathways. Knowledge of the regulation and function of p21 in normal and cancer cells has opened up several areas of investigation and has led to novel therapeutic strategies. The discovery in 1993 and subsequent work on p21 has illuminated basic cellular growth control, stem cell phenotypes, the physiology of differentiation, as well as how cells respond to stress. There remain open questions in the signaling networks, the ultimate role of p21 in the p53-deficiency phenotype in the context of other p53 target defects, and therapeutic strategies continue to be a work in progress. *Cancer Res; 76(18); 5189–91.* ©*2016 AACR.*

See related article by El-Deiry et al., Cancer Res 1994;54:1169–74.

Historical Context of the p21 Discovery

It has been just over 20 years since the discovery of p21 as a p53-regulated cell-cycle inhibitor (1, 2). At the time of the discovery made at the Johns Hopkins School of Medicine (Baltimore, MD), we were looking for mechanisms by which *p53* acts as a tumor suppressor gene. P53 had been recognized as a sequence-specific DNA-binding protein (3), and we had identified a genomic p53 response element (4). With the discovery of *p21(WAF1)* as a direct p53-regulated target gene, our early data showed that it suppresses tumor colony growth. Steven Elledge's laboratory, then at Baylor College of Medicine (Houston, TX), identified p21 as a CDK2-interacting protein from a yeast two-hybrid screen (5). Around the same time, David Beach's laboratory, then at Cold Spring Harbor Labs, identified p21 within mammalian cyclin–CDK protein complexes as a universal CDK inhibitor (6). Put together, the original discovery of p21 sheds light for the first time on the mechanism of a mammalian cell-cycle checkpoint. The phenomenon of a mammalian cell-cycle checkpoint had been suggested to involve p53 and another putative p53 target, *GADD45* (7). With the discovery of p21 and subsequent work in the field, it became clear that p21 was uniquely involved in maintaining the G_1 cell-cycle arrest when the checkpoint is triggered. Thus, mammalian cells exposed to stress, such as from radiation, DNA-damaging chemotherapy, or other stress, such as nutrient deprivation, trigger this p53/p21 G_1 checkpoint that causes growth arrest until conditions are more favorable, that is, the damage is repaired or the nutrients become available. It was recognized that initiation of the G_1 cell-cycle checkpoint involves rapid degradation of cyclin D1, but to maintain the checkpoint, p21 is required.

P21 Mutations and Knockout Mice

Early results demonstrated that p21 mutations are extremely rare in human cancer and that the mouse knockout of p21 shows cell-cycle defects but no tumor-prone phenotype (8). Data from The Cancer Genome Atlas suggest that p21 is not infrequently mutated in bladder cancer (Philip Abbosh, Fox Chase Cancer Center, personal communication). It is not clear why p21 is mutated in bladder cancer (and not other smoking-related cancers) and whether mutations impact tumor growth or patient prognosis.

P53-Independent Effects of p21

P21 was also discovered as Sdi1, a senescence-derived inhibitor, and as MDA6, a melanoma-derived antigen. P21 was subsequently shown to be expressed in postreplicative compartments *in vivo*, for example, in colonic crypt villi or muscle cells. *P21* is an exceedingly regulated gene with many pathways impacting on its gene promoter. The protein is also regulated by phosphorylation (e.g., Akt that promotes retention of p21 in the cytoplasm) or N-terminal ubiquitination. It became clear that p21 may serve a role to not only inhibit cyclin–CDK complexes but to also promote the assembly of these complexes, especially in the G_2–M phase of the cell cycle. Knowledge regarding p21 extended into physiologic roles in growth arrest associated with differentiation or stem cell renewal (9).

P21 Is Induced but Does Not Promote Apoptosis

Relationships between p21 and p53 function began to unravel after the original discovery. In the classic article published in *Cancer Research* in 1994 (2), we performed experiments documenting induction of p21 protein as a consequence of p53 activation. We showed that p21 induction occurs in cells that undergo p53-dependent cell-cycle arrest or apoptosis. At the time, it was becoming widely recognized that defective

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apoptosis is a hallmark of cancer, in addition to increased proliferation that had been observed for a couple of decades. Linking p21 induction to the p53 response in cells undergoing apoptosis further solidified the generality of its regulation by p53, including in the clearest situation of tumor suppression, that is, that was mediated by cell death or apoptosis when the tumor suppressor was activated. Demonstrating that p21 was not induced in p53-independent apoptosis tightened the link between p53 and p21 at least in response to agents that trigger p53 during cell death versus those that trigger cell death independently of p53. The p53–p21 axis quickly became accepted as a central pathway for cell-cycle control and tumor suppression. The data in the 1994 *Cancer Research* article demonstrated the cell cycle–inhibitory role of p21 at the protein level and evaluated the induction of p21 by p53 through Western blotting and immunocytochemistry. We showed in the classic article in *Cancer Research* (2) that p21 levels increase in cyclin E complexes in cells after DNA damage, and this was associated with inhibition of cyclin–CDK activity in phosphorylation of a histone substrate. Importantly, this only occurred if cells expressed wild-type p53 protein but not mutant p53, as often occurs in cancer. The relevance to cancer was clear at the time, that when the *p53* gene becomes mutated, the activation of p21 and its role in suppression of the cell-cycle machinery would be lost.

There are few times in one's scientific career where a major discovery is made that is clearly not incremental. Based on the recognition of the p53 DNA response element (4), we anticipated that p53 would specifically and directly bind and transcriptionally activate a number of genes that may explain its role as a tumor suppressor. We did not anticipate the simplicity of a gene encoding a protein, such as p21, that would directly through physical association inhibit the very machinery (cyclin–CDK complexes) of cell proliferation. At the time, simply understanding the elegance of this molecular regulation and the direct connection between a major tumor suppressor gene whose activities were poorly understood and the major drivers of the cell proliferation cycle was at the very least very inspiring. It was a great example of the convergence of evidence from different fields, such as biochemistry, cell biology, pathology, and cancer, using different tools and model organisms. One other comment to share the excitement in late 1993 was how quickly the authors of the 1994 *Cancer Research* article (2) collaborated to advance the field. This was before the time of big data, microarrays, NGS, or even loading controls in Western blots. The advances that were made shaped our understanding of cell growth control. The data have held up for more than 20 years and have been repeated hundreds if not thousands of times.

Subsequent work would evaluate activities of p53 in DNA repair or cell death more thoroughly. We found that p21-deficient cells are also repair deficient. Although the initial ideas may have correlated induction of p21 with cell death, more experiments suggested that p21-deficient cells are more prone to undergo apoptosis after DNA damage. Thus, p21 may protect cells from proapoptotic agents. Insights into the antiapoptotic or prosurvival function of p21 involved findings that p21 may localize to mitochondria and inhibit caspases and potentially other activities of p21 in the cytoplasm. Such observations have been used to explain why p21 may be infrequently mutated in cancer, as lack of p21 expression reduces cell survival, especially in the face of harsh DNA-damaging environments.

CDK Inhibition as a Cancer Therapeutic Strategy

P21 was the first mammalian CDK inhibitor to be described, and this was followed by the discovery of others that became classified into two families: the p21/p27/p57 family and the p15/p16/p18/p19 family of CDK inhibitors. It was recognized that the tumor-suppressing roles of p16 involved both CDK-inhibitory as well as activities of an alternative reading frame p14ARF. P14ARF became a focus of intense interest in the cancer research field due to its involvement in mediating p53 activation in response to oncogenic signals. The discovery of CDK inhibitors fueled efforts to develop small-molecule CDK inhibitors as therapeutic agents, and this continues to this day. For example, palbociclib, a CDK4/6 inhibitor, has been approved by the FDA as a treatment for breast cancer.

Targeting the Cell Cycle in Cancer Therapy

We have known about phases of the cell cycle and the G_1-phase restriction point for many decades. However, with the recognition of cell-cycle checkpoints in the early 1990s and the mechanisms that further unraveled, the insights opened up an avenue of investigation for oncologists who use combination therapies. With deeper understanding of the cell cycle and its regulation, it became possible to ask more sophisticated questions about drug interactions and the schedule dependence of drug activity. A drug that causes G_1 cell-cycle arrest given concurrently with a drug that acts in S-phase may not be such a good strategy, as the mechanism of one drug may interfere with the activity of the second drug. The unraveling of the cell-cycle checkpoints, in particular the p53-dependent p21-requiring cell-cycle checkpoint, after DNA damage also provided a rationale for the development of tumor-specific therapeutic strategies. A normal cell with an intact p53 G_1 checkpoint undergoes arrest in G_1 following exposure to a DNA-damaging agent, while a tumor cell proceeds to G_2, where it can be targeted for killing by a second drug designed, for example, to block checkpoint kinases active in the G_2 phase. Such strategies continue to be tested in ongoing clinical trials.

Does p21 Always Suppress Growth?

P21 expression has been observed in tumors, and it has been assumed that it is acting to restrain proliferation and tumor growth. From a tumor specimen, it is possible to observe $p21^+/Ki67^+$ cells but this, in and of itself, does not mean the cells are proliferating. Cell proliferation is a dynamic process that occurs over time, and unless one performs *in vivo* experiments with lineage tracing to demonstrate that a $p21^+/Ki67^+$ cell actually divides, it is difficult to interpret the meaning. DNA synthesis *per se* as measured by BrdUrd incorporation *in vivo* or in cells in culture may be due to DNA repair or replication. Another approach may involve asking directly about patient prognosis if one looks at $Ki67^+$ tumor cells and then includes a proportion of $p21^+/Ki67^+$ cells. Recent results by Gorgoulis and colleagues have suggested that prolonged p21 expression in cell culture in the absence of wild-type p53 (in the background of tumor cells) may promote re-replication that could contribute to aneuploidy in such tumor cells. Whether such cells adopt more aggressive, invasive, or drug-resistant phenotypes is unknown, and the signals that activate p21, including TGFβ, *in vivo* in tumor models can certainly be further explored. Further discussion of the regulation

and function of p21 in growth control and DNA replication can be found in a comprehensive review (10).

Insights into p53-Dependent Tumor Suppression

Understanding the mechanism of p53 in tumor suppression has important implications for patients and cancer therapy. This extends to associations of p53 status in tumors and prognosis, to resistance or sensitivity to specific therapeutic agents, to using the insights and signaling pathways to develop novel drugs. With the discovery of p21 and its regulation by p53, a paradigm was established for further work in the p53 field that followed. Although there are numerous p53 activities in cells, various interacting proteins and multiple inputs, one aspect of our understanding of downstream signaling was impacted by the p21 discovery. The control of p21 by p53 through sequence-specific DNA binding of the *p21(CDKN1a)* gene and subsequent cell biological effects at the time provided the clearest evidence for how p53 functions to suppress cell growth and within the checkpoint to allow time for repair prior to DNA replication. The paradigm allowed further work to identify other transcriptional targets of p53 and solidified our understanding that the transcriptional function of p53 is fundamental to its role as a tumor suppressor. From our own work perhaps, TRAIL death receptor 5 (DR5) is an example of insight into how p53 activation leads to cell death and cancer suppression *in vivo*. We learned quickly that unlike the unique role of p21 in being critical for the G_1 cell-cycle checkpoint, the cell death pathway is more complex. Multiple genes are induced, such as *bax*, *puma*, *noxa*, and others, while some are repressed. Much like CDK inhibitors emerged as a therapeutic strategy, knowledge of p53′s proapoptotic targets, such as DR5 and TRAIL, led us to develop ONC201 that is currently in multiple clinical trials.

It is clear that our insights regarding p53 and its transcriptional targets that link directly to cellular phenotypes in growth arrest, cell death, DNA repair, etc., beginning with p21, helped move the field forward over the last two decades. Imagine if the primary target of p53 function was a miRNA whose targets were unclear and whose function was obscure 20 years ago. The fact that p21 was recognized as a CDK inhibitor linked it to the cell-cycle machinery and a concrete mechanism of cancer suppression. The relationship of p53 to p21 induction after cellular stress has provided an initial experiment for many student and postdoctoral fellows interested in studying cancer or developing novel therapies. This has helped numerous scientists gain confidence in their ability to replicate a reproducible experimental finding.

Future Directions for the Field

Efforts continue to understand how p53 suppresses cancer. Although some efforts to knock out one or two p53 transcriptional targets *in vivo* have failed to recapitulate the p53 deficiency phenotype, no study has attempted to inactivate a larger number of targets. I believe it remains very plausible that if greater numbers (half a dozen or a dozen or more) of important p53 targets are inactivated *in vivo*, this will increase the chances for recapitulating the tumor-prone phenotype of p53 deficiency. I believe such work should have some priority as the alternative involves many expensive and ongoing approaches in many directions while there is compelling evidence from the best understood mechanisms. There is much to be learned still about the tissue specificity of the p53 response *in vivo*, including with regard to the role of p21. Therapeutics that utilize the targets of p53, such as CDK inhibitors or proapoptotic genes, hold promise, as do insights and further work optimizing therapy combinations to achieve the greatest synergies.

Disclosure of Potential Conflicts of Interest

W.S. El-Deiry has ownership interest (including patents) in Oncoceutics and p53 Therapeutics. No other potential conflicts of interest were disclosed.

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