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Cell Cycle News & Views

Autophagy and tumor cell invasion

Comment on: Macintosh RLTP, et al. Cell Cycle 2012; 11:2022–9;
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Ravi K. Amaravadi; Abramson Cancer Center and Department of Medicine; Perelman School of Medicine; University of Pennsvylania; Philadelphia, PA USA;
Email: ravi.amaravadi@uphs.upenn.edu; http://dx.doi.org/10.4161/cc.22147

Of the hallmarks of cancer,[1] the process of invasion and metastases is arguably the least well-understood and therefore least drugable. Invasion through the extracellular matrix is arguably the critical distinction between carcinoma in situ and carcinoma with metastatic potential. Efforts to understand targetable mechanisms of cancer invasiveness are sorely needed. In a recent issue, Macintosh et al. provided evidence that autophagy, the degradative process by which cells sequester and recycle cytoplasmic components, plays a role in tumor cell invasion.[2] Using a glioma cell line expressing a doxycycline-inducible shRNA directed against the essential autophagy gene ATG12, the effects of autophagy inhibition in two-dimensional (2D) culture and a three-dimensional (3D) organotypic culture system was studied. Knockdown of ATG12 had no significant impact on many cellular functions in 2D culture, including profileration, vaibility and migration. However, knockdown of ATG12, compared with non-target knockdown, resulted in a reduced capacity to invade an organotypic matrix consisting of human fibroblasts embedded in polymerized collagen (**Fig. 1**). Since efforts are underway to target autophagy therapeutically in cancer,[3] the authors conclude that autophagy represents a drugable mechanism of tumor cell invasion.

This study provides further evidence that autophagy can contribute to the malignant phenotype of cancer cells and could be a therapeutic target in certain cancers. The results also underscore a recurring theme in autophagy research, that cellular phenotypes of autophagy modulation are more pronounced within the tumor microenvironment than in traditional 2D culture. In an ovarian cancer model, acute activation of the ARHI tumor suppressor gene induced autophagic cell death in vitro but autophagic cell survival in vivo.[4] In a study of aggressive and indolent melanoma cell lines, elevated autophagy levels did not correlate with proliferation or invasion in 2D culture, but did correlate with invasion of a collagen matrix in 3D culture and tumor growth rate in vivo.[5] Together with the work by Macintosh et al., there is a growing body of evidence that while 2D culture can provide important insights into autophagy's role in cancer, experiments done within a 3D model system that includes elements of the tumor microenvironment are critical to understanding the functional consequences of autophagy modulation.

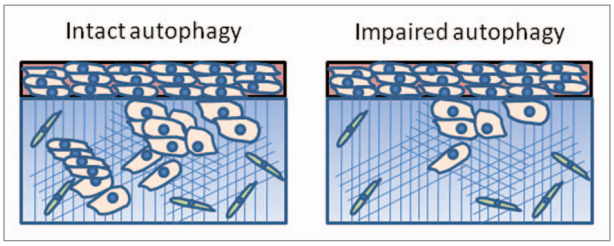

Figure 1. Autophagy contributes to tumor cell invasion. In a 3D oragnotypic culture system consisting of glioma cells (pink) collagen matrix (blue) containing embedded fibroblasts (green), glioma cell invasion of the extracellular matrix was reduced in cells deficient in the essential autophagy gene ATG12.

Can impairment of other components of autophagy produce reduced invasion? Currently, the only autophagy inhibitor that is in clinical trials is hydroxychloroquine, which blocks the lysosome.[3] While ATG12 is not currently a druggable target, emerging autophagy inhibitors may be able to target other components of autophagic vesicle assembly. Testing the effects of pharmacological autophagy inhibitors targeting proximal and distal components on tumor cell invasion will be important. It will also be critical to test the effects of genetic and pharmacological autophagy inhibition on a larger number of cell lines from a wider variety of malignancies.

Understanding the mechanism by which autophagy regulates tumor cell invasion will also be equally important. Tumor cell invasion is a complex phenomenon that includes diverse molecular pathways including secretion of metalloproteases, generation of lipid signaling molecules, signaling through integrin-associated kinases, such as focal adhesion kinase (FAK), and massive rearrangement of cytoskeleton.[6] Interestingly, autophagy has been implicated in many of these processes already. For instance, autophagy has been shown to be required for secretion of certain proteins[7] and regulation of sphingosine derivatives.[8] Finally, an intimate link has been found between autophagy and FAK signaling. One of the essential autophagy genes is FIP (FAK interacting protein) 200,[9] and, more recently, reduced FAK signaling, which occurs in a dynamic manner during invasion, was shown to be a strong inducer of cytoprotective autophagy.[10]

It will be important to understand how to capitalize on autophagy's role in promoting tumor cell invasion. Additional studies in models of advanced disease including in vivo studies will be required to understand the role of autophagy inhibitors as cancer invasion inhibitors. Future work will be needed to determine if the transition from premalignant noninvasive cells to malignant invasive cancer cells also relies on autophagy. If this were the case, autophagy inhibitors could not only be effective chemotherapeutic, but also chemoprevention agents.

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p53-Aurora A mitotic feedback loop regulates cell cycle progression and genomic stability

Comment on: Wu C-C, et al. Cell Cycle 2012; 11:3433–42; PMID:22894933; http://dx.doi.org/10.4161/cc.21732

Cheng-Ming Chiang; Simmons Comprehensive Cancer Center and Department of Biochemistry and Department of Pharmacology; University of Texas Southwestern Medical Center; Dallas, TX USA; Email: cheng-ming.chiang@utsouthwestern.edu; http://dx.doi.org/10.4161/cc.22113

p53 is a tumor-suppressor protein regulating cell cycle progression, apoptosis, senescence, autophagy, metabolism, stem cell differentiation and inflammatory responses. Its function, as a genome guardian sensing DNA damage and acting primarily as a transcription factor to control gene activity, is often deregulated in cancer cells by genetic mutation or guilt association with unintended viral and cellular factors that alter p53's gene targets, compartmentalization, protein stability or posttranslational modification state. An important function of p53 in mitotic progression that has been previously noted—abnormal centrosome amplification, leading to chromosome segregation defects—was first observed in p53-null mouse embryonic fibroblasts.[1] However, the molecular mechanism underlying p53-regulated centrosome formation remains elusive. In a recent issue of *Cell Cycle*, Wu et al. identified Aurora A kinase normally associating with centrosomes as a functional target of p53 occurring at both transcriptional and post-transcriptional levels.[2] The abundance of Aurora A transcripts in wild-type p53-containing cells could be regulated by RNA polymerase II-dependent transcription via the p53-pRb-E2F3 pathway (**Fig. 1**, pathway 1). When p53 is downregulated or inactivated by oncogenic signaling, transcription from the cell cycle inhibitor p21 gene is suppressed, leading to upregulated cyclin-dependent kinase 2 (CDK2) activity that phosphorylates pRb and releases E2F3 transcription factor, which, in turn, activates

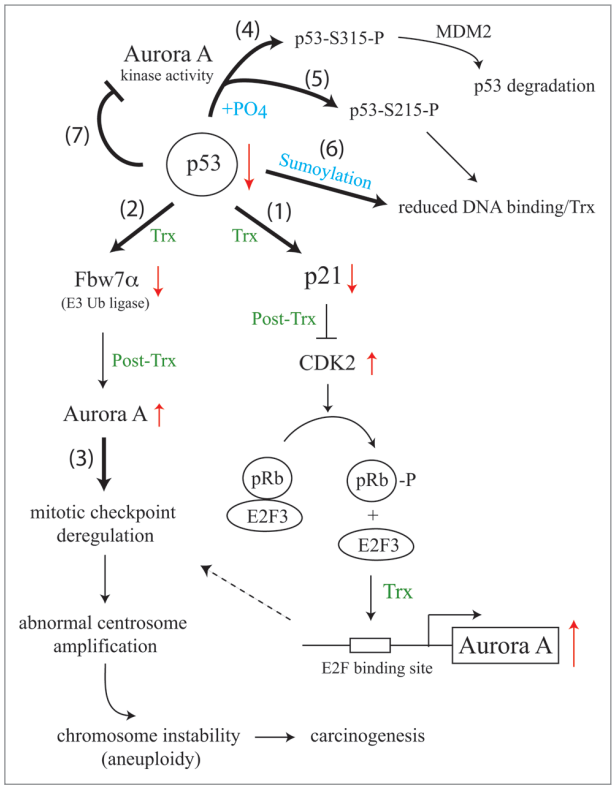

Figure 1. p53-Aurora A mitotic feedback loop regulating p53 and Aurora A function. Distinct pathways modulating p53 and Aurora A activity at the transcriptional (Trx) or post-transcriptional (Post-Trx) level are depicted by thick lines, numbered one to seven, with P indicating phosphorylation (PO4) and up and down red arrows reflecting upregulated and downregulated protein or RNA amounts, respectively.

Aurora A gene transcription. Interestingly, reduced p53 level also diminishes transcription from another p53 target gene encoding the Fbw7α component of an E3 ubiquitin ligase that degrades Aurora A, resulting in an increased amount of Aurora A protein at the

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post-transcriptional level (**Fig. 1**, pathway 2). A significant increase of Aurora A RNA and protein by concurrently inducing p53-pRb-E2F3 and p53-Fbw7α pathways causes mitotic checkpoint deregulation, abnormal centrosome amplification, chromosome segregation defects, aneuploidy and eventually carcinogenesis (**Fig. 1**, pathway 3).

While p53 inversely regulates the protein level of Aurora A by both transcriptional and post-transcriptional pathways, the kinase activity of Aurora A also provides a feedback mechanism to regulate the protein stability and DNA-binding activity of p53 by phosphorylating p53 at serine 315, triggering MDM2-induced p53 degradation (**Fig. 1**, pathway 4) or by phosphorylating p53 at serine 215, blocking its DNA-binding activity (**Fig. 1**, pathway 5).[3,4] Inactivation of p53 by Aurora A-mediated phosphorylation at the residue corresponding to serine 215 of human p53 is essential for maintaining self-renewal and pluripotency of mouse embryonic stem cells,[5] a scenario analogous to cancer cell growth triggered by overexpression of Aurora A and loss of p53 function. Phosphorylation-blocked p53 binding to DNA at serine 215 by Aurora A downregulates p53 target gene transcription, similar to the effect of PIAS family proteins-mediated sumoylation at lysine 386 of p53, which also inhibits the DNA-binding activity of p53 and suppresses its transcriptional activity (**Fig. 1**, pathway 6).[6] Intriguingly, p53 can reciprocally inhibit the kinase activity of Aurora A by interacting with the N-terminal region of Aurora A to block the enzymatic activity of Aurora A residing in its C-terminal region,[7] perhaps via allosteric regulation (**Fig. 1**, pathway 7). Clearly, a fine balance between counteracting Aurora A and p53 function is critical for driving mitotic progression in normal cells. Paradoxically, a complete loss of p53 protein, as seen in p53-null mice, leads to frequent deletion or downregulation of Aurora A loci,[8] further highlighting the essence of maintaining a functional p53-Aurora A feedback loop in mitotic cell cycle control. Considering that Aurora B kinase can also phosphorylate p53 and lead to its degradation,[9] some extent of functional redundancy likely exists in Aurora family proteins as well, even though their subcellular localizations appear different throughout cell cycle progression.

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Cell-autonomous circadian DNA damage response: Is the case closed?

Comment on: Gaddameedhi S, et al. Cell Cycle 2012; 11:3481–91; PMID:22918252; http://dx.doi.org/10.4161/cc.21771

Roman V. Kondratov; Center for Gene Regulation in Health and Disease; BGES Department; Cleveland State University; Cleveland, OH USA; Email: r.kondratov@csuohio.edu; http://dx.doi.org/10.4161/cc.22115

The role of the circadian clock in cancer development and progression is intimately linked to the role of the circadian system in genotoxic stress response.[1] A few original reports (reviewed in ref. 2) support the idea that disruption of the circadian clock may cause the development of cancer. Several key cancer-related genes and signaling pathways have been found to be potential targets for the clock. Circadian clock-dependent regulation of proliferation, cell cycle, apoptosis, DNA damage response and DNA repair have been proposed as potential molecular mechanisms.[3] Circadian clock proteins, specifically the periods, have been added to the list of tumor suppressors.[2] However, with a growing number of experimental works, more and more clouds are appearing in an originally clear sky of the circadian clock/cancer connection. No effect of circadian disruption on the rate of tumorigenesis has been reported in model systems, and in some cases, even the opposite effects were observed.[3] It is necessary to mention that different groups were using different model systems, different experimental approaches and worked with different circadian clock genes, which adds to the growing discrepancy in the literature. Recent attempts to investigate the effect of several circadian clock genes on tumorigenesis in the same study[4] has also been challenged;[5] therefore, questions about the clock and cancer are still open. In agreement with the role of genotoxic stress response in cancer, original in vivo and in cell culture reports (reviewed in refs. 1 and 2) demonstrated the regulation of DNA damage-associated pathways by the clock proteins, but later reports (reviewed in ref. 3) made the picture more complicated.

In the present paper by Gaddameedhi and collegues,[6] the authors made an attempt to study, systemically, the role of the circadian clock proteins in the DNA damage response. The authors used three different DNA damaging agents, which produce three different types of DNA damage and activate three different response pathways. Cells deficient for the components of the positive arm of the molecular circadian clockwork (*Clock* and *Bmal1*) and of the negative arm (*Periods* and *Cryptochromes*) have been treated with the indicated agents; cell survival, the effects on cell cycle checkpoints, apoptosis and DNA repair were all assayed. With minor exceptions, the authors did not find any significant difference between wild-type and circadian clock-deficient cells. Together, with recently published works[7,8] on the sensitivity of *Crptochrome1,2*$^{-/-}$ and *Bmal1*$^{-/-}$ cells to DNA damaging agents, the present work argues that in contrast to the observed difference in sensitivity to genotoxic agents in vivo, there

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is no effect of circadian disruption in the cell-autonomous response.

What is the reason for the difference between in vivo and in cell culture-based experiments? One possibility suggested by the authors is that in culture, the cells lose circadian rhythms very fast, due to the loss of synchronization between cells. Another interpretation, also discussed by the authors, is that the absence of circadian clock-dependent control of systemic factors, such as growth factors or hormones, is an inherent difficulty of cell culture, and may affect the response to genotoxic stress. There is at least one more interpretation—tissue specificity of the response. In most of the experiments,[6-8] the authors used mouse embryonic or adult fibroblasts; fibroblasts are one of the most popular models to study DNA damage response and results and conclusions obtained with this system have been often extrapolated as universal. However, fibroblasts are not a major contributor to an organism's sensitivity to genotoxic stress in vivo. Lymphatic and epithelial tissues are two major sites of damage, which determine sensitivity in vivo. Tissue specificity of the response to DNA damage upon circadian clock disruption has been suggested as a potential interpretation of existing conflicting results.[3] Another possibility is the difference in the effect on normal and transformed cells. The authors partially addressed this by demonstrating the absence of any effect of genetic manipulation with *Per1* expression in cancer cell lines NCI-H460 and HCT-116. But suppression of *Bmal1* expression in the same HCT-116 cell line resulted in an increased resistance to irradiation.[9] Therefore, to "close the case," it is necessary to study the consequences of circadian disruption on the response to DNA damage in cells of epithelial and lymphatic origin, fibroblasts in vivo and tumor cells.

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Transcriptional profiling of apoptosis: Cell death classification moves toward the systems era

Comment on: Galluzzi L, et al. Cell Cycle 2012; 11:3472–80; PMID:22918244; http://dx.doi.org/10.4161/cc.21789

Zhi Yao[1] and György Szabadkai[1,2,*]; [1]Department of Cell and Developmental Biology; Consortium for Mitochondrial Research; University College London; London, UK; [2]Department of Biomedical Sciences; University of Padua; Padua, Italy; *Email: g.szabadkai@ucl.ac.uk; http://dx.doi.org/10.4161/cc.22116

In a recent issue of *Cell Cycle*, Galluzi et al.[1] describe comparative gene expression profiles of the cellular response to three different stressors, with the striking conclusion that cisplatin (CDDP)-induced apoptosis does not rely on direct effects either on mitochondrial integrity or nuclear transcriptional reprogramming due to DNA damage. While their approach gives important insight into the wide range of possibilities a cell can use to cope with stress at the transcriptional level, the paper also highlights the challenges to define cellular death pathways at the systems level.

Classifying cell death modalities dates back to the times when the most powerful approach was thorough morphological observation by light and electron microscopy in the '70s, allowing us to limit the catalog of cell death to merely three types.[2] In the following decades, with the introduction of biochemical approaches and of the series of novel stressor substances tested, the picture became always fuzzier and more complex. The latest effort to categorize the regulated death pathways listed 13 modalities, defined by the combination of at least 30 unique biochemical processes and their sensitivity to numerous pharmacological and genetic modifiers.[2] Recognizing the complexity of the systems involved, modeling based on the fairly well-characterized principal protein components of cell death pathways (considering apoptotic, regulated necrotic and autophagy pathways) and posttranslational biochemical modifications gained importance.[3] Finally, further realizing that the cell death pathways depicted by classical biochemical studies might just scratch the surface, in recent years a series of studies applied large-scale, unbiased gene expression and proteomic approaches to identify novel players in cell fate determination. These studies were particularly boosted by the need to identify novel targets in the pharmacological treatment of drug-resistant cancer.[4]

While many cellular stress pathways impinge on gene expression (e.g., unfolded protein response and endoplasmic reticulum stress), modulation of transcription is envisaged to be the most relevant in defining the cellular response to genotoxic stress, which directly targets the integrity of DNA. Indeed, a recent meta-analysis of a large set of gene expression profiles underlying the cellular response to ionizing radiation-induced double-strand breaks confirmed the central role of the transcriptional targets of p53, mediating DNA repair and survival, senescence or programmed cell death.[5] In contrast, the study by Galluzzi et al.,[1] using CDDP, which rather causes intra- and interstrand links as the primary mechanism of DNA damage,[6] showed that the enriched transcriptionally modified pathways belong to classes not directly associated with cell death induction, particularly when compared with C2-ceramide and CdCl2, classic inducers of mitochondrial apoptosis. The finding was corroborated by the limited modulation of CDDP-induced reduction of clonogenicity in genetically modified yeast clones, lacking several components of the apoptotic pathway. The study accompanies a previous effort by the same group,[7] where, using genome-wide shRNA profiling, they identified a set of genes that are able to inhibit or enhance the toxicity caused by

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CDDP (CDDP response modifiers, CRMs). While it would have been expected that the primary target of the CDDP-induced transcriptional stress response will include this gene set, strikingly, only about 10% of the CRM genes were found significantly up or downregulated at the transcriptional level in the present study. This led to the cautious conclusion of the authors that it is likely that regulation at the translational and posttranslational levels is the essential factor in triggering the actual cell death execution machinery following CDDP treatment. Accordingly, the transcriptional regulation accompanying CDDP toxicity is either negligible or responsible for only secondary adaptive stress responses. Indeed, recent studies suggested that DNA damage can cause massive changes in global translational profiles,[8] and the direct interaction of CDDP with a wide range of other cellular components could give rise to extensive posttranslational modification.[9]

Notably, the work illustrates the power of combining bioinformatic and experimental approaches to identify new transcriptional targets in the DNA damage response network, but also indicates the formidable challenges when aiming to define and classify cell death pathways based on unbiased genome-wide systems analyses. It will certainly take a lot of effort to get there, but now it also appears achievable if the combined approaches of high-content imaging, transcriptomic and proteomic techniques will be adopted by a larger community of cell stress-focused research groups.

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Wee1-Hsp90 inhibitor combination treatment: Molecular therapy with potentially broad applicability

Comment on: Iwai A, et al. Cell Cycle 2012; 11:3649–55; PMID:22935698; http://dx.doi.org/10.4161/cc.21926

Vinata B. Lokeshwar; Division of Urology Research; Department of Urology and Cell Biology; University of Miami Miller School of Medicine; Miami, FL USA; Email: vlokeshw@med.miami.edu; http://dx.doi.org/10.4161/cc.22119

Pharmacological inhibition of molecular chaperone Hsp90 is an attractive approach for anticancer therapy, since the chaperone activity of Hsp90 is critical for the stability and activity of a variety of cellular client proteins. The list of Hsp90 client proteins is always expanding and includes transcription factors, steroid hormone receptors, protein kinases, oncogenes, proto-oncogenes and signaling molecules.[1,2] Since many of these client proteins promote tumor growth, metastasis and angiogenesis, inhibition of Hsp90 can be the "one punch" that cripples the tumorigenic and metastatic potential of tumors regardless of their tissue or cellular origin. Hsp90 inhibitors have been combined with a variety of chemotherapy and targeted treatment drugs, but the rationale for such combinations is largely empirical. The study by Iwai et al. not only demonstrates potent synergistic antitumor activity upon combining a Wee1 kinase inhibitor and several Hsp90 inhibitors, but the rationale for simultaneous inhibition of Wee1 kinase and Hsp90 is based on the elegant mechanistic data that have been previously published by this group.[3-5]

Wee1 is a cell cycle-dependent kinase that is essential for the G_2-M checkpoint. While hyperactivity of Wee1 kinase causes cell cycle arrest in the G_2-M phase, its inhibition causes premature mitotic entry and cell death.[6] Wee1 is a client protein of Hsp90. More importantly, Wee1 (Swe1 in yeast) kinase phosphorylates a conserved tyrosine residue in Hsp90 (Y38 in human Hsp90α; Y24 in yeast Hsp90).[4,5] Wee1 targets and phosphorylates Hsp90 while it is in an "open" conformation, and reversible phosphorylation is important for its ability to chaperone a number of clients, including several oncogenic kinases.[3-5,7] Further, Hsp90 inhibitors bind less efficiently to phosphorylated Hsp90, hence inhibition of Wee1 enhances drug binding to Hsp90 and makes cells more sensitive to Hsp90 inhibitors. The discovery of Wee1-mediated phosphorylation of Hsp90 and its functional consequences provides the rationale for combining a Wee1 inhibitor with an HsP90 inhibitor as a novel anticancer combination therapy.

This group previously reported that pharmacological inhibition of Wee1 and its molecular silencing with siRNA uniformly increased apoptotic activity of the Hsp90 inhibitor 17-AAG in vitro.[5] The current study by Iwai et al. demonstrates that Wee1 inhibition synergizes with any one of several clinically evaluated Hsp90 inhibitors to inhibit cell growth in yeast and in an androgen-independent and invasive human prostate carcinoma cell line, PC3.3 The fact that 17-AAG, SNX-2112 and STA-9090 (ganetespib) are currently in clinical trials and synergize with a Wee1 inhibitor (Inhibitor II) suggests that Wee1-Hsp90 inhibitor combination therapy may be translatable to the clinic. One of the intriguing features of the study is that the drug combination not only inhibits Wee1 activity, but also transcriptionally downregulates it. It is possible that in a feedback loop, the drug combination causes sustained downregulation of Wee1, which, in turn, achieves sustained tempering of Hsp90 chaperone activity and downregulation of several Hsp90-dependent signaling pathways related to protection from apoptosis and DNA damage. The unique gene signature and enhanced apoptotic signaling induced by Wee1 inhibitor/Hsp90 inhibitor combination can be further confirmed by combining an Hsp90 inhibitor with another Wee1-inhibitor (e.g., MK-1775).[6] Nevertheless, the Iwai et al. study shows that combined Wee1-Hsp90 inhibition may be an effective targeted therapy based on clearly defined molecules that are critical for cancer growth and progression.

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Consistent with this theme, the PC3 xenograft study corroborates the in vitro observations regarding the potent antitumor activity of this drug combination and confirms the molecular events responsible for the observed enhanced antitumor activity. Clinical applicability of such a strategy is substantial, since effective targeting of Hsp90 should be efficacious against a variety of tumors and high expression of Wee1 is associated with poor disease-free survival in certain cancers.[8] Further, based on the reported gene signature, this drug combination may also synergize with standard DNA damaging drugs. Therefore, future studies to evaluate bioavailability, toxicity, dosing sequence and schedule are needed to support clinical trials of this mechanism-based novel combination therapy.

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Cisplatin-induced apoptosis and development of resistance are transcriptionally distinct processes

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Theocharis Panaretakis; Department of Oncology-Pathology; Cancer Centre Karolinska; Karolinska Institutet; Stockholm, Sweden; Email: theoharis.panaretakis@ki.se; http://dx.doi.org/10.4161/cc.22114

Cisplatin is a cornerstone treatment for numerous malignancies, including lung cancer. Despite the extensive research on the mechanisms by which this platinum compound exerts its potent anticancer effects, the predominant mode of action is unclear. It was originally thought that cisplatin inflicts mainly DNA damage, leading to cancer cell death.[1] Additional research revealed that cisplatin can also kill cancer cells that lack their nucleus, indicating that the targeting of cytoplasmic molecules is equally effective.[2,3] Genetic or epigenetic disruption of any of these pathways or cellular adaptation mechanisms to this toxic agent is likely to lead to the development of resistance. In fact, resistance to cisplatin is the most frequent clinical outcome despite the initial beneficial anticancer effects induced by this drug. In a recent issue of *Cell Cycle*, Galluzzi et al., utilized multi-faceted approaches in an attempt to answer some of the aforementioned questions.[4] One of these approaches involved a comprehensive analysis of the transcriptional signatures elicited by cisplatin. The findings of this array provide further support to the notion that cisplatin-induced apoptosis is largely transcription-independent. However, the adaptation of the cancer cells to the cytotoxic insults inflicted by cisplatin and the ensuing development of resistance may depend on the expression of key proteins. The authors found that two genes that may confer resistance were found to be transcriptionally upregulated by cisplatin. The transcription of PDXK, an enzyme involved in vitamin B6 metabolism and required for optimal cisplatin cytotoxic responses, is downregulated in A549 cells.[5] Another gene that is transcriptionally downregulated by cisplatin is DHRSX, an oxidoreductase that may mediate the lethal effects of cisplatin by the generation of cytotoxic reactive oxygen species. The most significantly upregulated gene is RRAD, a Ras-related GTPase. The functional significance of this induction in RRAD gene expression is not clear. RRAD has been shown to promote apoptosis by activating the p38 MAPK and by decreasing the expression of Bcl-xL.[6] Importantly, RRAD gene expression is epigenetically downregulated in prostate cancer clinical samples.[7] The mechanisms behind the transcriptional regulation of these proteins are not known and deserve further investigation. Overall, this study provides novel insights on the cell death signaling cascades induced by cisplatin as well as on mechanisms of resistance, exploitation of which may improve the efficacy of this anticancer drug in the clinic.

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