MEKing Retinoids Work Better

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DOI 10.1016/j.ccr.2010.07.007

Retinoids can induce terminal differentiation in a subset of neuronal cancer cells, but what determines response has been unclear. In a recent issue of *Cell*, Hölzel and colleagues show that hyperactive RAS signaling imparts retinoid resistance to neuroblastoma cells, which can be reversed by inhibiting MAPK, suggesting new therapeutic opportunities.

Retinoids are natural or synthetic analogs of vitamin A that have shown promise as cancer therapeutics. These molecules bind to the retinoid acid receptors (RARs) and retinoid X receptors (RXRs) and mediate gene transcription in a ligand-dependant manner through retinoic acid response elements (Altucci and Gronemeyer, 2001). As a seminal example of retinoid-based therapies, acute promyelocytic leukemia (APL), a disease defined by chromosomal translocations that lead to the production of aberrant RARα fusion proteins resulting in a blockade of normal myeloid cell differentiation, can be effectively eradicated by treatment with all-trans retinoic acid (ATRA) combined with chemotherapy (Fenaux et al., 2001). Although ATRA is now a mainstay of APL therapy, the retinoids have mainly been investigated as potential chemopreventative agents, and there are no other examples where retinoids have proven useful in treating established diseases. In addition, it should be emphasized that the experience in APL is part of the emerging theme of combinatorial strategies as a key component of maximally exploiting molecularly targeted therapies.

Neuroblastoma is an embryonal tumor of the developing autonomic nervous system that exhibits diverse clinical behaviors ranging from spontaneous regression to relentless progression. Survival for children with high-risk neuroblastoma ($\sim$50% of cases) has shown only modest improvement despite dramatic escalations in the intensity of cytotoxic therapy over the past two decades, but there have been recent advances in improving outcomes with targeted therapies designed to eradicate minimal residual disease after chemotherapy (Maris, 2010). It has been known for decades that retinoids can drive terminal differentiation of some neuroblastoma cells in vitro, but strikingly many cell models show complete resistance to the effects of retinoids. Despite the observation of heterogeneity in response to retinoids, extensive preclinical evaluation of these compounds led to a phase 1 trial of isotretinoin (13-*cis* retinoic acid) in neuroblastoma patients after myeloablative consolidation, showing it was well tolerated with a hint of antitumor activity (Villablanca et al., 1995). The efficacy of isotretinoin was then tested in a phase 3 randomized trial with a factorial design after a randomization to myeloablative or continuation chemotherapy. The cohort of patients assigned to receive posttransplant therapy with isotretinoin had a modest but significantly improved 3 year event-free survival probability of 46% ± 6% versus 29% ± 5% (p = 0.027) (Matthay et al., 1999), although this was even more marginal on further follow-up (5 year EFS = 42% ± 5% versus 31% ± 5%; p = NS) (Matthay et al., 2009). Subsequently, addition of an immunotherapy regimen targeting the cell-surface ganglioside GD2 with cytokines further improved outcome (A.L. Yu et al., 2009, J. Clin. Oncol., abstract), thus the combination of isotretinoin with immunotherapy is now considered a standard approach to eliminating rare residual tumor cells that may survive intensive cytotoxic therapy.

In a recent issue of *Cell*, Hölzel and colleagues reported the identification of potential modulators of sensitivity to retinoid therapy by using an RNAi-based loss-of-function genetic screen (Hölzel et al., 2010). Here, they specifically address the clinical problem that although isotretinoin appears to be an active agent, it fails to prevent relapse in a significant percentage of neuroblastoma patients. In a series of well-controlled experiments, the investigators identified *NF1* as the lead candidate gene for influencing retinoic acid-induced differentiation in neuroblastoma cell models, with depletion of *NF1* resulting in loss of retinoid-induced differentiation capacity. This group recently showed that ZNF423, a component of the RAR/RXR transactivator complex, is essential for the response to retinoids in neuroblastoma (Huang et al., 2009). Hölzel connected these observations through the MEK-ERK component of the MAPK signaling network. The authors showed that hyperactive Ras results in suppressed expression of *ZNF423*, and thus decreased transcription of retinoic acid response genes (Figure 1). Although MAPK signaling has been implicated in modulating retinoid responsiveness previously, Hölzel and colleagues provide a very clear link between common cancer driving mutations such as in ras hyperactivation and/or receptor tyrosine kinase mutations and insensitivity of cells to retinoid therapy.

There are several important clinical implications related to these findings, some of which are tested in the Hölzel et al. paper. The observation that *NF1* was the top hit in the siRNA screen led to the search for mutations in primary neuroblastoma samples. Although the authors did find evidence for inactivating mutations and homozygous deletions, these were rare, consistent with past reports (The et al., 1993). Ongoing full-genome sequencing efforts as part of the neuroblastoma TARGET project (http://target.cancer.gov) will further

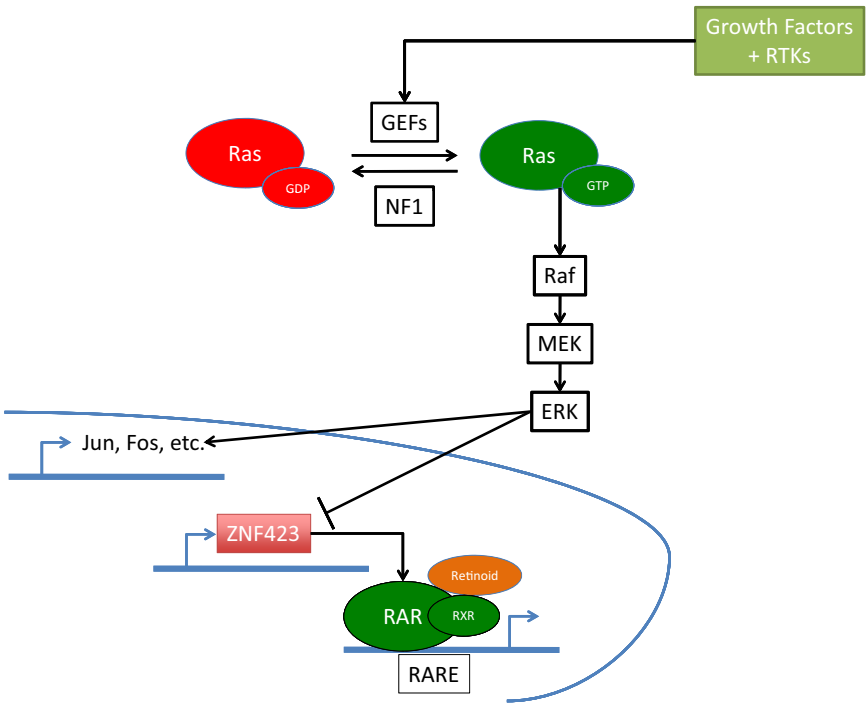

Figure 1. The Extracellular Signal-Related Kinase Component of the Mitogen-Activated Protein Kinase Pathway

The extracellular signal-related kinase (ERK) component of the mitogen-activated protein kinase (MAPK) pathway is depicted in simplified form. Activation of this pathway occurs with hyper-activated ras through a variety of mechanisms, including loss of the NF1 tumor suppressor that keeps ras locked into the GTP-bound (active) state, as well as through signaling through receptor tyrosine kinases (RTKs) via guanine exchange factors (GEFs) such as SOS. Canonical ERK signaling promotes cellular proliferation via transcriptional regulation of multiple transcription factors such as Jun and Fos. Although MAPK signaling has a multitude of additional effects in a cell context-specific manner, it is now clear that at least in neuroblastoma cells, activated MAPK signaling suppresses the transcription of ZNF423, a key component of the retinoic acid receptor complex, thus rendering cells insensitive to the effects of the retinoid ligands that need to bind to the RAR/RXR/ZNF423 coactivator complex to drive transcription at retinoic acid response elements (RAREs).

define the frequency of genetic and epigenetic inactivation of *NF1* in neuroblastoma, as well as the frequency of other alterations that may converge on MAPK pathway hyperactivation. In addition, the authors explore *NF1* and *ZNF423* as prognostic biomarkers. They show convincingly that low mRNA expression of both genes is associated with a significantly decreased patient survival probability. Although this may not be too surprising because low *NF1* and *ZNF423* reflects hyperactivated Ras/MAPK, and the clinical utility of stratifying traditional therapy based on these mRNA signatures is far from certain, the findings do suggest that certain neuroblastomas are hardwired to be resistant to retinoid therapy from the time of diagnosis and that NF1 and ZNF423 expression levels may be a robust predictor of responsiveness to isotretinoin therapy. Finally and perhaps most importantly, Hölzel and colleagues take the first steps in exploring whether their findings might be leveraged therapeutically. The authors show that pharmacologic inhibition of MEK can sensitize neuroblastoma cells genetically engineered to be resistant to retinoid-induced terminal differentiation. Several MEK inhibitors are in development, and a dual MEK1/2 inhibitor recently showed tolerabiltiy and evidence for single agent antitumor activity in an early phase 1/2 clinical trial (J.R. Infante et al., 2010, J. Clin. Oncol., abstract).

Although the data presented by Hölzel and colleagues seem readily translatable, several important questions and challenges will need to be addressed before the work here can impact patient care. First, prospective validation of *NF1* and *ZNF423* mRNA levels as the most reliable and robust biomarker of retinoid responsiveness is required and will need to be compared to other potential biomarkers such as phospho-MEK. It will also be important to determine whether the markers of retinoid resistance are dynamic; i.e., is MAPK pathway activation also a mechanism of acquired resistance to isotretinoin therapy, or is it solely a collateral event of oncogenic mutations that are driving tumorigenesis? Serial measurement of *NF1* and *ZNF423* levels (or other markers) in tumor cells during the course of treatment and at relapse will be needed to understand how best to utilize this combinatorial approach in the clinic. It will also be critically important to determine early on how combined MEK inhibition with isotretinoin will fit into the overall neuroblastoma treatment strategy. Although isotretinoin alone was originally designed as a postcytotoxic therapy maintenance biotherapy, the big problem in neuroblastoma currently is achieving an initial quality remission. Indeed, there has been little advancement in improving chemotherapeutic remission induction, and no molecularly targeted agents have been incorporated into the initial phases of high-risk neuroblastoma therapy. Although integration of a MEK inhibitor with isotretinoin at the end of therapy may ultimately make the most sense, it may be possible to define a cohort of patients at diagnosis with tumors primed to respond to this combination therapy.

In a broader sense, this work clearly demonstrates the power of unbiased genetic screens to identify tractable therapeutic strategies for human cancer. By focusing on current clinical realities, such as the fact that isotretinoin is considered a standard therapy for neuroblastoma, but one with marginal clinical benefit, investigators can not only uncover novel insights into the mechanisms underlying human cancer, but also identify rapidly translatable hypotheses because they build on existing paradigms. This approach to targeted therapeutics has the potential to advance the field more rapidly than developing a drug target in a vacuum devoid of considering current clinical practice.

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EZH2 Mutations: Mutating the Epigenetic Machinery in Myeloid Malignancies

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DOI 10.1016/j.ccr.2010.07.006

Two recent studies identified loss-of-function mutations in the histone H3 methyltransferase EZH2 in myelodysplastic syndromes and myeloproliferative neoplasms, provided further demonstration of mutations in epigenetic modifiers in myeloid malignancies, and suggest *EZH2* functions as a tumor-suppressor gene in these malignancies rather than an oncogene as in some other malignancies.

Laboratory studies have implicated epigenetic dysregulation as a common pathogenetic mechanism in myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPNs), and acute myeloid leukemia (AML). Notably, genome-wide methylation studies have identified a set of genes recurrently targeted by aberrant promoter hypermethylation in AML (Figueroa et al., 2010). Recent studies have shown that chromosome translocations involving *MLL* and *JARID1A* found in myeloid malignancies result in dysregulation of chromatin state and resultant activation of genes that contribute to leukemogenesis (Krivtsov et al., 2008; Wang et al., 2009). In addition, hypomethylating agents (5-azacytidine and decitabine) are approved for the treatment MDS and AML, illustrating the therapeutic relevance of epigenetic alterations in myeloid malignancies. However, until recently, somatic mutations that directly dysregulate the epigenetic state of leukemic cells but are not chromosomal rearrangements had not been reported.

Two groups led by Nicholas Cross and Joop Jansen recently reported identification of recurrent somatic *EZH2* mutations in MDS, MPNs, and MDS/MPN overlap disorders (Ernst et al., 2010; Nikoloski et al., 2010). Alterations in the long arm of chromosome 7 are common in MDS and AML and are associated with adverse outcome of these patients. Both groups first used high-resolution SNP arrays to characterize regions of acquired uniparental disomy (aUPD) and microdeletions involving chromosomal locus 7q36, which includes the *EZH2* gene. They then performed gene resequencing of *EZH2* and identified somatic frameshift, nonsense, and missense mutations in MDS, MDS/MPN, and MPN, with and without concomitant chromosome 7 alterations. The identified missense mutations most commonly affected the CXC-SET domain, which is required for histone methyltransferase activity, or domain II, which is necessary for binding to SUZ12, of EZH2. Importantly, they did not identify *EZH2* mutations in AML with chromosome 7 abnormalities, including those with chromosome 7 aUPD. These results suggest the existence of an additional, not yet identified, tumor suppressor gene for MDS, MPN, and AML on chromosome 7. This is consistent with previous cytogenetic studies of chromosome 7 deletions in MDS and AML that have delineated several regions of common deletion on chromosome 7, only one of which includes the *EZH2* locus (Le Beau et al., 1996). In addition, both current studies suggest that patients having MDS with *EZH2* mutations have worsened overall survival compared to those having MDS without *EZH2* mutations, independent of the presence or absence of cytogenetic abnormalities involving chromosome 7.

The genetic data implicating *EZH2* in the pathogenesis of myeloid malignancies is in contrast to that observed in other malignancies. A recent study employed whole transcriptome resequencing to identify somatic *EZH2* mutations in lymphoma and found that all mutations affected a specific residue within the EZH2 SET domain, tyrosine 641 (Morin et al., 2010). However, tyrosine 641 was not identified as a mutational hotspot in MDS and MPN by Cross, Jansen, and colleagues. Moreover, previous studies