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Flipping the phenotypic switch on a novel antimelanoma differentiation strategy

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In the 1859 *Origin of Species*, Charles Darwin defined his theory of evolution as the process by which genetic variation can naturally select individuals with a phenotypic advantage to survive and reproduce within a changing environment. This simple, yet powerful philosophy not only influenced our current understanding of species evolution, but also provided insight into the complexity of cancer drug resistance. As humans, not all cancer cells that make up a tumor are the same. For instance, the genetic heterogeneity associated with malignant melanoma can give rise to cellular subpopulations with distinct combinations of properties such as invasiveness, self-renewal, proliferation, and differentiation. Activating mutations in driver oncogenes (BRAF and NRAS) is a common genetic event associated with the malignant transformation of melanocytes into melanomas. Molecular-targeted therapies against these oncogenes and their associated pathways, although initially promising, have ultimately proven to be of limited clinical efficacy due to the ability of cellular subpopulations to adapt to (or ‘evolve’) and resist the stress induced by treatment. Thus, alternative therapeutic approaches are desperately needed to bypass unwanted drug resistance, perhaps by targeting all cellular subpopulations within melanomas irrespective of their genetic heterogeneity. Here, we summarize recent findings published in *Cancer Cell* by Saez-Ayala and colleagues, who believe that the key to discovering such an elusive therapeutic strategy lies in understanding and manipulating melanoma cell heterogeneity itself. Coaxing a heterogeneous population of melanoma cells toward homogeneity was accomplished by inducing a cellular switch to a more differentiated phenotype.

“the key to discovering [a] therapeutic strategy lies in ... manipulating melanoma cell heterogeneity itself”

Mounting evidence has shown that the molecular events regulating melanocyte differentiation share similarities to events driving progression of malignant melanoma. Non-pigmented melano- blasts located in the neural crest can migrate and differentiate into pigmented melanocytes found in the epidermis, hair follicle, inner ear, or retina. The heterogeneous fate of melanocyte differentiation is highly dependent on the expression levels of the master pigment gene regulator microphthalmia-associated transcription factor (MITF) (Hoek and Goding, 2010). High MITF expression dictates the proliferative and

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differentiated phenotype of melanocytes in the epidermis and hair follicle bulb. Conversely, MITF-negative melanocytes located in the hair follicle bulge are non-pigmented and take on a self-renewing stem cell-like phenotype. Similar to melanocyte differentiation, MITF can also function as a 'rheostat' by regulating the variable phenotypic properties of melanoma cells (Carreira et al., 2006; Cheli et al., 2012). As MITF expression increases, melanoma cells shift from a senescent, invasive, stem cell-like phenotype to a more differentiated, proliferative phenotype. A 'phenotype-switching' model has been proposed to explain how the invasive properties of melanoma subpopulations can be reversible dependent on MITF gene programs regulated by the surrounding tumor microenvironment (Hoek and Goding, 2010). This observation has led Saez-Ayala et al. to pursue a unique two-step therapeutic strategy that would (i) reverse the metastatic potential of melanoma cells by inducing differentiation through MITF upregulation and (ii) specifically target the elimination of MITF-differentiated melanoma cells. Importantly, the efficacy of this therapeutic approach would not be affected by the presence of specific melanoma driver mutations or drug resistance mechanisms.

Methotrexate (MTX) is an antifolate drug widely used as a cancer therapeutic, which functions by inhibiting dihydrofolate reductase (DHFR) activity and depleting intracellular pools of deoxythymidine triphosphate (dTTP). Although melanomas are naturally MTX resistant, Saez-Ayala et al. identified MTX as a potent inducer of MITF mRNA and protein in human SK-MEL-28, mouse B16/F10, and other melanoma cell lines. Consistent with the 'rheostat model', MTX-induced MITF expression suppressed melanoma invasiveness and promoted differentiation as shown by the induction of differentiation target genes and melanoma cell dendricity. Tyrosinase is among the MITF-responsive genes induced by MTX and plays a key role in synthesizing melanin pigment within melanosomes. MTX induces pigmentation within melanoma cells, and co-localization of tyrosinase with a stage II melanosome marker was observed. Taken together, the ability of MTX to reverse the invasive phenotype of melanoma cells by promoting MITF-dependent differentiation represents the first step of the authors' two-step melanoma therapeutic strategy.

Next, Saez-Ayala and colleagues sought to exploit MTX-induced tyrosinase expression to selectively eradicate the differentiated melanoma cells. The authors previously synthesized a catechin-derived compound, 3-O-(3,4,5-trimethoxybenzoyl)-(-)-epicatechin (TMECG), as a second-generation antifolate therapy with enhanced bioavailability and efficacy in melanomas (Sanchez-Del-Campo et al., 2008). TMECG has similar antifolate properties compared with MTX. However, TMECG was designed as a tyrosinase-activated prodrug that can target melanomas in a cell type-specific manner. Tyrosinase catalyzes the oxidation of TMECG into its active quinone methide form (TMECG-QM), which acts as an irreversible competitive inhibitor of DHFR (Sanchez-Del-Campo et al., 2008). The authors hypothesized that TMECG would be an ideal candidate drug to target MTX-differentiated melanoma cells. In support of the two-step therapeutic strategy, combination MTX/TMECG treatment enhanced both growth inhibition and apoptosis of melanoma cells. MTX/TMECG treatment also suppressed the growth of two patient-derived melanoma cell lines that were mutant for both BRAF and MEK1 and resistant to BRAF-targeted therapy (PLX-4720). The mechanism of synergy observed with MTX/TMECG treatment in melanoma was explained by enhanced dTTP depletion, which can trigger DNA damage and subsequent S-phase

accumulation. The authors demonstrated that the combination therapy can also elevate the expression of the proapoptotic protein p73 (TP73) in SK- MEL-28 cells in an E2F1-dependent, p53-independent manner.

Although MTX/TMECG combination therapy demonstrated impressive efficacy in culture, the true success of any therapeutic strategy lies within its in vivo efficacy. Because melanoma is known to be a highly immunogenic malignancy, and it is unclear how tumor immunosurveillance would influence MTX/TMECG efficacy, Saez-Ayala et al. employed a syngeneic mouse model in which B16/F10 melanoma cells were subcutaneously injected into immunocompetent C57BL/6 mice. A significant reduction in tumor growth was observed following TMECG treatment alone relative to vehicle control; MTX alone had no effect. This observation could be explained by the activation of TMECG through basal levels of tyrosinase in B16/F10 tumors. Importantly, the combination of MTX and TMECG was synergistic in the ability to block proliferation and induce apoptosis. In vivo quantification of bioluminescence signals from luciferase-tagged B16/F10 cells validated the effect of MTX/TMECG on overall tumor burden. The authors also used this imaging technique to illustrate the ability of combination therapy to potentially suppress the dissemination of B16/F10 cells from the spleen into the liver. Note that these observations are consistent with the decreased invasiveness of human SK- MEL-28 cells observed following MTX-induced MITF expression. Collectively, the MTX/TMECG two-step therapeutic strategy was shown to be effective in inhibiting melanoma cell invasiveness and survival within both in vitro and in vivo systems.

Saez-Ayala and colleagues addressed two potential concerns that could arise if MTX/TMECG is to be considered for future clinical trial development. First, melanoma subpopulations remaining after MTX/TMECG treatment could eventually acquire resistance, as commonly occurs with many targeted therapies. This is especially concerning because the tumor burden observed following combination therapy is significantly reduced, but not completely eradicated. To address issues of potential therapeutic resistance, Saez-Ayala et al. dissociated luciferase-tagged B16/ F10 tumors cells from vehicle or MTX/TMECG-treated mice to investigate their continued sensitivity to MTX/ TMECG treatment in vitro. Strikingly, cells harvested from both vehicle and MTX/TMECG-treated mice were equally sensitive to the combination therapy in culture. However, it still remains to be seen whether melanoma cells can escape MTX/TMECG-induced apoptosis in vivo following prolonged treatment. Second, the authors addressed a potential concern over the toxicity that MTX/ TMECG may have on surrounding tissues. The goal of this combination therapy is to specifically target the death of MITF-expressing melanoma cells; however, there are non-melanoma MITF-expressing cells in the body (e.g., pigmented eye epithelial cells, skin melanocytes) that could be susceptible to TMECG-induced apoptosis. However, the authors observed no deleterious effects of MTX/TMECG on the integrity of skin melanocytes or pigmented epithelial cells of the eye following extended exposure to MTX and TMECG. This is most likely because depletion of dTTP should only affect proliferating cells; the RPE and differentiated melanocytes in the skin and hair follicle are non-proliferating. This same dosing schedule also caused no weight loss in the treated mice.

As the human species continues to evolve, so do the complexities of the diseases we suffer from. Drug resistance has been a long-standing impediment in our battle against melanoma, as well as many other malignancies. Decades ago, we learned that cancer cells can respond to initially efficacious chemotherapy with a multidrug resistant phenotype (e.g., through amplification of MDR1). More recently, tumors were found to recur after highly effective molecularly targeted therapies, often through secondary mutations in the same gene, or in associated pathway members. The extensive cellular heterogeneity of many tumors is thought to be responsible for much of the observed acquired resistance. In this report, Saez-Ayala and colleagues switch from the popular targeted approach by introducing a novel bi-modal, differentiation-based therapy that first attempts to homogenize tumor heterogeneity, followed by treatment with a drug that specifically targets the newly differentiated tumor cells. This two-step therapeutic strategy has the potential to revolutionize the manner in which we treat drug-resistant cancers. Ultimately, most of the functions of tumor cells rely on hardwired pathways that were part and parcel of the normal cells from which they originated. Perhaps exploiting these same hardwired pathways, such as differentiation, will be key to future therapeutic break-throughs, keeping us a step ahead of the ever-evolving tumor.

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