Reinforcing targeted therapeutics with phenotypic stability factors

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Reinforcing targeted therapeutics with phenotypic stability factors
Paul Yaswen

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Reinforcing targeted therapeutics with phenotypic stability factors

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Deregulated cell cycle progression can often be traced to intrinsic defects in specific regulatory proteins in cancer cells. Knowledge of these primary defects has led to targeted approaches that exploit the defects and spare normal cells. However, the success of such targeted approaches is still hit-or-miss. Genetic and epigenetic variability inherent in most tumors often results in phenotypic heterogeneity that, in turn, results in *de novo* or acquired resistance to therapeutic agents. The ability of cells to compensate and adapt to the inhibition of a specific cell cycle mediator is not remarkable. What is novel and of great potential importance is that the ability of cells to exhibit such adaptability varies markedly. "Phenotypic stability factors" that restrict the ability of cells to undergo epithelial-mesenchymal transitions (EMT) may dictate the success or failure of targeted therapies by interfering with compensatory changes such as deregulation of CDK2 activity. Identification of existing and new agents that induce and maintain phenotypic stability factors will inform and enable synergistic approaches to the eradication of even the most aggressive tumors.

Keywords: cancer therapy, cyclin-dependent kinase (CDK), drug resistance, epithelial-mesenchymal transition (EMT), inflammation, Notch, NF-κB, Ovol, TGFβ

Abbreviations: CSC, cancer stem cell; CDK, cyclin-dependent kinase; EMT, epithelial-mesenchymal transition; ER, estrogen receptor; GSI, γ-secretase inhibitor; HER2, Human Epidermal Growth Factor Receptor 2; NICD, Notch intracellular domain.

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Introduction

Adaptation is an understudied yet overtly important aspect of cancer progression that ultimately leads to treatment failure and mortality. In this perspective, a new strategy is described to combat the evolution of drug resistant growth by cancer cells. This strategy stems from experimental data indicating that cultured cells vary considerably in their abilities to undergo adaptive growth when challenged with a therapeutic drug. Although a number of reports have linked drug resistance with epithelial-mesenchymal transitions (EMT) and "cancer stem cells (CSCs)," the reasons remain obscure why these phenotypic traits are more or less prevalent in particular cancers.

Resistance to targeted therapies is tied to changes in cell cycle regulators

Targeted therapy often involves the use of agents that specifically inhibit portions of signaling pathways associated with growth of particular cancers. Examples of targeted therapies for breast cancers include tamoxifen, trastuzumab (Herceptin), and more recently palbociclib (PD0332991). Anti-estrogen therapy, such as tamoxifen, is most beneficial in patients whose breast cancers express estrogen receptors (ER), with 49% of ER-positive patients responding to such therapy.[2] Trastuzumab interferes with the Human Epidermal Growth Factor Receptor 2 (HER2). Approximately 20% of aggressive breast cancer tumors express high levels of HER2 and respond to trastuzumab therapy.[3] Palbociclib specifically targets cyclin-dependent kinases CDK4 and CDK6,[4] and is currently in clinical trials in patients whose tumors express the Retinoblastoma (Rb) protein–a tumor suppressor which arrests cell replication unless inactivated by CDKs.[5] A common consequence of all these targeted therapies is the arrest of tumor cells in G1 phase of the cell cycle, along with distinctive morphological and biochemical changes characteristic of cellular senescence.[4,6,7]

While upstream mediators of the senescence response vary with stimuli, they generally converge on the Rb pathway. In cycling cells, Rb is maintained in a partially or fully inactive state by the action of CDKs. According to the canonical model of cell cycle regulation, Cyclin D

complexed with CDK4 or CDK6 phosphorylates Rb, resulting in initial activation of the transcription factor E2F-1, whose target genes are responsible for progression into S-phase of the cell cycle. Subsequent activation of Cyclin E/CDK2 further phosphorylates Rb and completes entry into S-phase.[8] Inhibition of Cyclin/CDK complexes by endogenous CDK inhibitors such as $p16^{Ink4A}$, $p21^{Cip1}$, or $p27^{Kip1}$, results in Rb activation, forcing cells into G1-arrest.[9] Palbociclib mimics p16 by directly inhibiting CDK4/6, resulting in G1 arrest of Rb-positive cancer cells. Tamoxifen and trastuzumab indirectly cause decreased levels of Cyclin D, and increased expression of p21 and p27–which inhibit CDK2 activity,[6,7] also resulting in G1 arrest. Thus data suggest that targeted therapies commonly rely on inhibition of CDK4/6 and/or CDK2 activity for effective treatment of cancer patients.

Targeted therapeutics are not effective for all cancer cells. Approximately 85% of HER2-amplified and 40% of ER(+) advanced breast cancers show *de novo* resistance to therapeutic targeting.[3,6,10,11] In addition, approximately 50% of ER(+) breast cancer patients treated with anti-estrogenic compounds, such as tamoxifen, show acquired resistance in their lifetimes.[12-14] Tumors with acquired tamoxifen resistance are reported to have lost p21 expression[15] or inactivated p27.[16] Likewise, many HER2 overexpressing tumors responsive to targeted therapy with trastuzumab treatment acquire resistance within 1 y[3] Finally, while palbociclib in combination with the aromatase inhibitor, letrozole, significantly prolonged progression-free survival compared with letrozole alone in women with ER(+), HER2(-) breast cancer, overall survival was not significantly improved.[17] Clinically, low p27 expression[18] and high levels of Cyclin E[19] are indicators of poor prognosis in breast cancer patients. Thus, acquired resistance to targeted therapeutics in breast cancer patients is a regular occurrence with the common theme being de-regulation of CDK2 activity.

In vitro systems have been used to gain better understanding of molecular mechanisms responsible for *de novo* and acquired resistance to targeted therapeutics. In cultured cells, induction of trastuzumab-resistance is linked to increased CDK2 activity through Cyclin E overexpression,[20] and decreased expression of p27.[3] Reports also indicate that reduction in Cyclin E levels leads to increased sensitivity to trastuzumab or chemotherapy in breast cancer cell lines,[20,21] while increased p27 expression in trastuzumab-resistant breast cancer cells also restores sensitivity.[3] Chemical inhibition of CDK2 activity in Cyclin E overexpressing, trastuzumab-resistant cells induces G1 arrest, suggesting that such cells are still dependent on CDK2 activity. In addition, CDK2 inhibition drastically diminishes anchorage-independent growth of human cancer cells and cells transformed with various oncogenes.[22]

Resistance to palbociclib is also associated with constitutive CDK2 activity. Induced palbociclib resistance in breast cancer cell lines resulted in decreased levels of endogenous CDK2 inhibitors, p21 and p27.[13] In one study, 25% of the human breast cancer cell lines analyzed (12/47) were resistant to palbociclib-induced senescence and consisted primarily of ER-negative, basal-type breast cancer cells, which tend to be more aggressive and less responsive to therapy clinically.[1] Further analysis demonstrated that in several palbociclib resistant lines, Rb continued to be expressed and phosphorylated in the presence of palbociclib. These data suggest that in some cell lines, phosphorylation/inactivation of Rb can occur independently of CDK4/6 activity. A comparison of published transcriptional profiles of palbociclib sensitive and resistant cell lines indicates decreased expression of Cyclin D and increased expression of the endogenous CDK4/6 inhibitor, p16, in the resistant lines (**Fig. 1**). However, transcript levels of Cyclin E, which complexes with CDK2, were generally increased. The finding that even some pre-malignant human mammary epithelial cells overcome palbociclib exposure quickly and exhibit evidence of deregulation of CDK2, p21, and p27 in response to extended CDK4/6 inhibition[13] indicates that resistance can occur in the absence of rare mutational events.

Altogether, these data indicate a role for deregulated CDK2 activity in mediating *de novo* or acquired resistance to several targeted therapies in breast cancer patients. Although the canonical pathway for cell cycle progression in adult cells involves the sequential activation of CDK4/6 followed by CDK2, others and we have found evidence that increases in CDK2 activity, due to decreased levels of CDK2 inhibitors and/or increased levels of Cyclin E, can circumvent the need for CDK4/6. Despite these findings, small molecule inhibitors of CDK2 have not fared well clinically, leading to speculation that increased levels of other CDKs or E2F activity in cancer cells may compensate for the requirement for CDK2 activity.[5,23]

Differences in adaptability are likely to correlate with the relative abilities of cells to undergo EMT

The ability of cells to compensate and adapt to the inhibition of a specific cell cycle mediator is not remarkable. What is novel and of great potential importance is that the ability of different cell lines to exhibit such adaptability varies markedly. Certain breast cancer cell lines show a striking inability to overcome palbociclib induced growth arrest, even when Rb expression is inhibited,[13] in accordance with our own recent findings that in some instances Rb family members p107 and p130 can mediate growth arrest in the absence of Rb.[24] Other cell lines easily overcome arrest, despite the presence of intact Rb. These differences in adaptability may be mechanistically linked with the relative abilities of specific cell lineages to undergo EMT.

The EMT process can be regulated by a diverse array of cytokines and growth factors whose activities are dysregulated during malignant tumor progression. Many of these cytokines and growth factors are already subjects of vigorous research, and specific small molecule inhibitors are in various stages of pre-clinical and clinical development. For example, the TGFβ and Notch signal transduction pathways have been shown to play central roles in the EMT process, and drug candidates with some clinical validation are already available. However, a rational framework for their effective use has yet to be determined.

The cytokine TGFβ is thought to play an essential role in the induction of EMT during cancer progression. TGFβ binds and activates TGFβR receptors; these in turn phosphorylate and activate downstream cytoplasmic molecules (e.g., Smad 2 and 3). Phosphorylated Smad 2 and 3 can in turn bind to Smad 4 and enter the nucleus, where they form complexes with other factors and promote the expression of several target genes related to proliferation, differentiation, apoptosis and cell migration. The consequences of TGFβ signaling are determined by cross talk with other signaling pathways; as a result, TGFβ can promote or inhibit cell proliferation even in different normal contexts.[25] In normal breast epithelial cells, TGFβ causes upregulation of CDK inhibitors and growth arrest. However in mesenchymal cells and most tumor cells, TGFβ causes increased CDK2 activity and promotes cell proliferation. Recent data implicate NFAT and c-Myc transcription factors in the switch from TGFβ mediated growth inhibition to promotion.[26] In such cells, palbociclib inhibition or shRNA-mediated knockdown of CDK4 can increase Smad transcriptional activity, and enhance rather than inhibit proliferation.[27] In some cases, this counterintuitive effect of palbociclib can be blocked by a TGFβRI kinase inhibitor.[27] These findings indicate that, despite its ability to suppress growth of many cancer cells, palbociclib can have the opposite effect in cancer cells in which TGFβ signaling pathways associated with EMT have been activated.

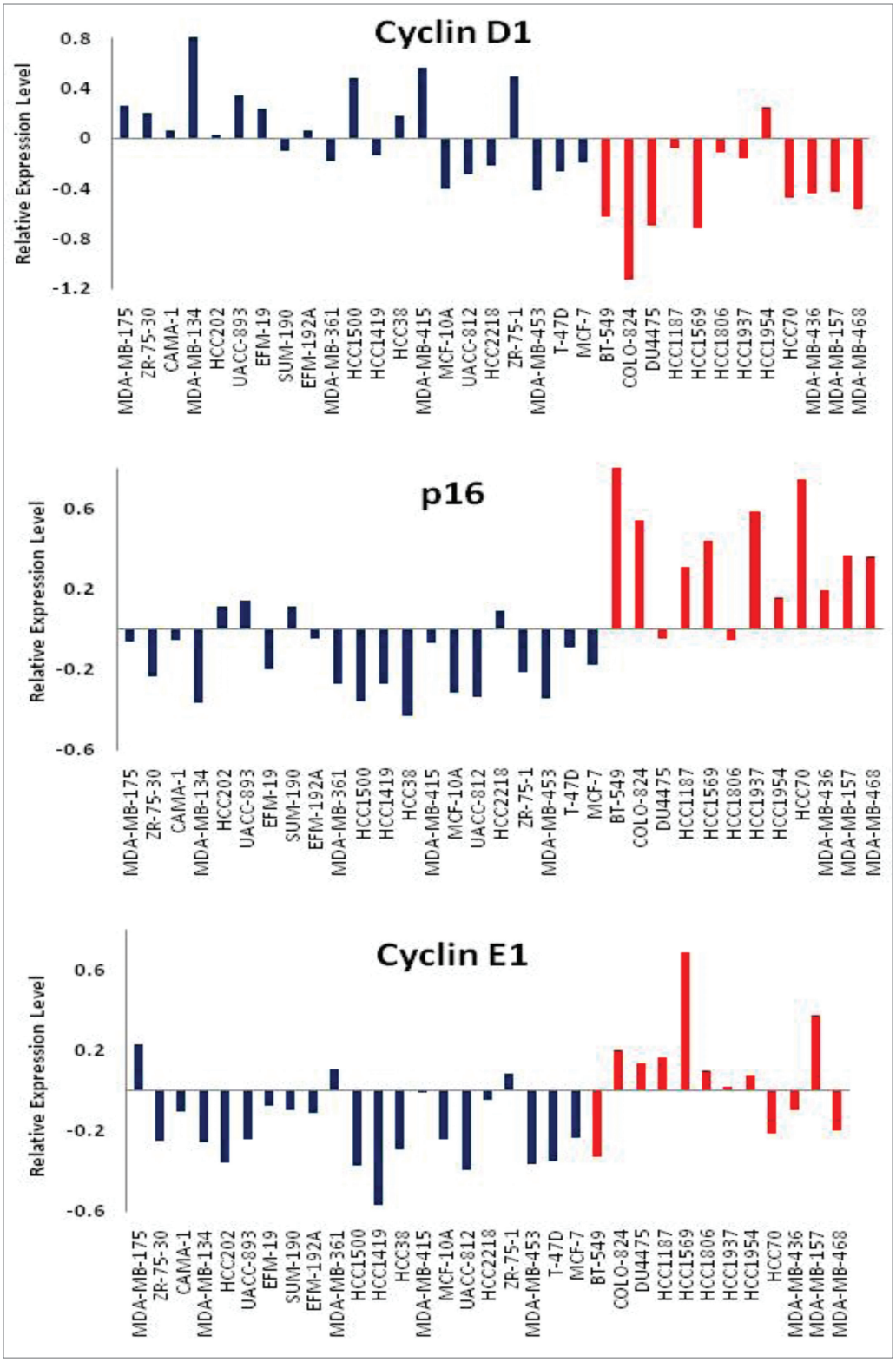

Figure 1. Consistent differences in the levels of Cyclin D and E mRNAs are observed in breast cancer cell lines resistant to palbociclib. Relative expression of indicated mRNAs in human breast cancer cell lines arranged from lowest to highest GI50 value for palbociclib. Cell lines are color coded by sensitivity: blue, palbociclib-sensitive; red, palbociclib-resistant. Adapted from. [1]

Another gene that has been linked to both EMT and resistance to targeted therapeutics is Notch1. The generic Notch pathway has been associated with regulation of mammary gland cell fate at several distinct developmental stages, and has been implicated in breast cancer initiation and progression.[28-30] There are 4 Notch receptors (Notch1, 2, 3, and 4) and 5 ligands (Delta-like-1 or DLL-1, 3, 4 and Jagged1 and 2).[31] Notch signaling is activated by direct cell–cell contact involving ligand-receptor binding, which is followed by proteolytic cleavage of the Notch intracellular domain (NICD), yielding the active form of the Notch receptor. NICD cleavage products subsequently translocate to the nucleus where they act as transcription factors activating the HES/HEY gene family.[32] By interacting with co-repressors or by sequestering transcriptional activators, HES and/or HEY proteins regulate the transcription of key genes including cell-cycle regulators p21 and cyclin D1, ubiquitin ligase SKP2–which promotes p27 degradation, transcription factors c-Myc and NF-κB2, and growth factor receptor HER2.[33] Notch signaling has been reported to maintain CSCs and increase EMT in breast cancer cell lines.[34] Although a number of genetic and pharmacologic approaches are either available or theoretically possible to block Notch signaling at different levels of the pathway, γ-secretase inhibitors (GSIs) originally developed as potential inhibitors of the presenilin γ-secretase complex that cleaves β-amyloid peptide (which may lead to Alzheimer disease through plaque formation) are the furthest in development as potential anticancer agents. Treatment of HER2(+) breast cancers with trastuzumab has been shown to result in a compensatory increase in Notch1-mediated proliferation, while combining Notch inhibitors LY411575 or MRK-003 with trastuzumab significantly reduced tumor recurrence in xenograft models.[35] Thus, treatment strategies that reduce the ability of tumor cells to undergo EMT also interfere with their ability to exhibit adaptive growth.

Adaptive growth can be limited by phenotypic stability factors

TGFβ and Notch pathway inhibitors may limit tumor plasticity through induction and/or maintenance of phenotypic stability factors (**Fig. 2**). Such factors may include known EMT inhibitors, including Elf5, GATA3, GRHL2, Klf4, and the miR200 family of microRNAs. One candidate for a master phenotypic stability factor is Ovol2A, a nuclear protein that functions as a master suppressor of almost all known EMT-inducing transcription factors by directly binding to their promoters. Ovol2A has exhibited ability to reprogram metastatic breast cancer cells back to an epithelial state.[36] In addition, there is good correspondence between levels of Ovol2A transcripts and palbociclib sensitivity in the limited number of human breast cell lines in which both have been measured.[1,36] Furthermore, Ovol2A expression is positively correlated with the overall and metastasis-free survival of postoperative breast cancer patients.[36]

While Ovol proteins have been described as active gatekeepers that prevent mesenchymal transdifferentiation and maintain epithelial identity, little is known about the regulation of the Ovol proteins themselves. Normally, epithelial cells may retain the ability to downregulate Ovol proteins under conditions of stress and remodeling, when migration of epithelial progenitors is critical for proper tissue reconstitution. If this hypothesis is correct, then mediators of tissue damage responses such as inflammatory cytokines and NF-κB are likely to be involved in Ovol protein regulation. Such inflammatory pathways are known to be chronically activated through positive feedback loops in a large percentage of cancers, and to be required for the malignant phenotype.[37] Furthermore, the growth promoting effects of Notch1 have been shown to be mediated by the NF-κB signaling pathway.[38] If activation of NF-κB is associated with downregulation of phenotypic stability factors such as Ovol2A, this will provide proof-of-principal for screening existing well-tolerated anti-inflammatory agents to determine whether they are capable of blocking the effect. More than 700 NF-κB inhibitors with varying specificities, including common aspirin, have been described,[39] and may be useful when combined with targeted therapeutics.

Well tolerated doses of new and existing agents that activate and maintain phenotypic stability factors such as Ovol2A are likely to act synergistically with therapies that target cell cycle regulators such as CDK2/4/6 to block the growth and recurrence of many types of human cancers. The prospective clinical significance of such synergism is that lower (and thus less toxic) drug levels will be needed, and the combined therapies will eliminate EMT-associated

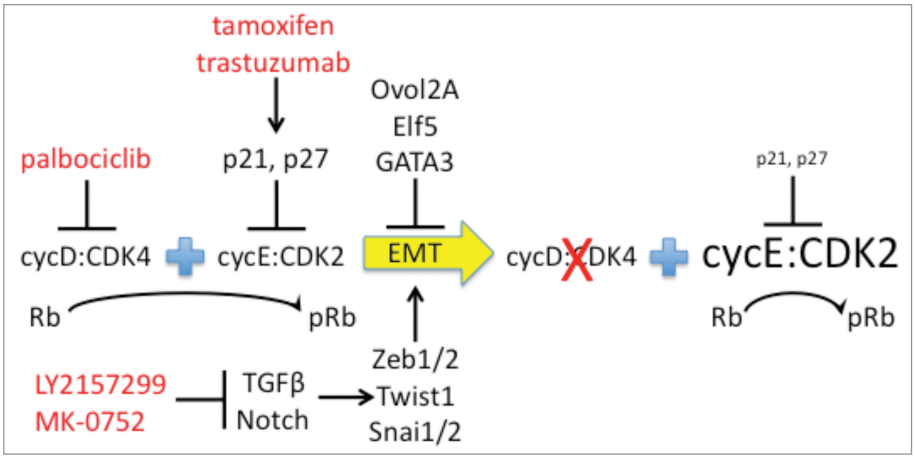

Figure 2. Scheme depicting hypothetical changes in cell cycle regulation accompanying EMT and acquired resistance to targeted therapeutics. Induced or constitutive down regulation of phenotypic stability factors (e.g., Ovol2A) is postulated to lead to decreased dependence on cyclin D/CDK4 for phosphorylation and inactivation of Rb family proteins leading to autonomous G1-> S transit.

cancer stem cells responsible for recurrence and metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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