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"Identification of Rb Phosphorylation Sites that Regulate Epithelial to Mesenchymal Transition and Invasion in Breast Cancer."

An elevated level of phosphorylation (hyperphosphorylation) of the Retinoblastoma tumor suppressor protein (Rb) is commonly present in most cancer cell types. The hyper-phosphorylation of Rb on 15 separate amino acids found in most human cancer promotes tumorigenesis. In fact, treatments that block Rb phosphorylation (cdk4/6 inhibitors) have recently been developed and approved to treat breast cancer patients (1). While particular Rb phosphorylation sites have been assigned specific functions in the regulation of proliferation and apoptosis, to date no specific information regarding the sites of Rb phosphorylation that regulate EMT (epithelial to mesenchymal transition) and invasion has been identified. In the studies described in this proposal we plan to investigate the role that specific Rb phosphorylation sites play in EMT and invasion, important components of tumorigenesis. EMT can be measured by evaluating the change in expression of epithelial cell proteins to mesenchymal proteins. Invasion assays are used to measure the ability of cells to move through an extracellular-matrix like material. Thus, we aim to use phosphorylation-site mutants of Rb and EMT reporter cell lines to elucidate the functions of Rb phosphorylation sites in EMT and invasion in breast cancer.

The specific questions to be addressed will be:

1. What sites of Rb phosphorylation are modified during EMT? Can dephosphorylation of Rb block the induction of EMT or stimulate the reverse MET (mesenchymal to epithelial transition)?

2. What is the effect of Rb phosphorylation on Rb binding activity to two proteins involved in invasion: Zeb1 and Sin1?

The preliminary data from our previous studies in breast cancer show that two treatments that effect Rb phosphorylation differently (the CDK4/6 inhibitor Palbociclib and phosphatase activation) cause different effects on invasion. We propose that the effects on invasion may be mediated by changes in site-specific phosphorylation of Rb which may regulate phosphorylation-regulated association of Rb with Zeb1 or Sin 1. For question 1 above, first we will use CRISPR generated EMT reporter lines (ATCC) that when stimulated with an inducing agent, will induce EMT (or the reverse process, MET). We can identify the Rb phosphorylation patterns at each stage using quantitative immunoblotting with antibodies that recognize specific Rb phosphorylation sites. To identify specific sites of phosphorylation or dephosphorylation needed for EMT/invasion, we will utilize Rb phosphorylation mutants (a gift from our collaborator, Dr. Ioannis Sanidas, Harvard Medical School) expressed in cells carrying an Rb siRNA to silence endogenous Rb expression (2). These cells will be evaluated for EMT by immunoblotting and by Invasion assays. For question 2, Rb phospho-site mutants that carry a FLAG-tag will be expressed in cells engineered to silence endogenous Rb. Then complex association between the Rb mutant and Zeb1 and Sin1 will be detected using immunoprecipitation with anti-FLAG antibodies. These experiments will lead to a publication including student authors and provide preliminary data for future proposals.