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PP2A Regulation of Cyclin E in Breast Cancer: Therapeutic Applications

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14. ABSTRACT Cyclin E is a major determinant in breast cancer initiation, progression, and chemotherapy response. Overexpression of cyclin E1 occurs in approximately 50% of breast cancers and this phenotype is associated with increased tumor aggressiveness, metastasis, hormone-independent growth, and poor patient survival. However, the molecular mechanism responsible for cyclin E alterations in breast cancers is not understood. In this research project, we explore the potential involvement of the protein phosphatase PP2A in causing cyclin E abnormalities in breast cancer. This will be achieved by identifying the PP2A holoenzyme complex that regulates cyclin E, evaluate the effects of PP2A activity knockdown on breast cancer progression, and screen for small molecules that can selectively inhibit PP2A regulation of cyclin E to attempt to correct for these alterations in breast cancers.					
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YEAR 1 PROGRESS REPORT (W81XWH-11-1-0176)
PP2A Regulation of Cyclin E in Breast Cancer: Therapeutic Implications

INTRODUCTION: Cyclin E is a major determinant in breast cancer initiation, progression, and chemotherapy response [1]. Overexpression of cyclin E1 occurs in approximately 50% of breast cancers and this phenotype is associated with increased tumor aggressiveness, metastasis, hormone-independent growth, and poor patient survival [2]. However, the molecular mechanism responsible for cyclin E alterations in breast cancers is not understood. In this research project, we will explore the potential involvement of the protein phosphatase PP2A in causing cyclin E abnormalities in breast cancer. This will be achieved by identifying the PP2A enzyme complex that regulates cyclin E, evaluate the effects of PP2A activity knockdown on breast cancer progression, and screen for small molecules that can selectively inhibit PP2A regulation of cyclin E to attempt to correct for these alterations in breast cancers.

BODY: Year 1 concentrated on completing the tasks outlined in Aim1, which were designed to identify the composition of the PP2A holoenzyme that associates and regulates cyclin E phosphorylation. We have achieved all the tasks outlined in the approved Statement of Work (SOW).

Tasks 1-3 (Aim 1): We successfully created expression vectors for tagged cyclin E for use in MudPIT analysis to identify interacting proteins and potentially the PP2A holoenzyme that targets cyclin E in breast cancer cells. The PP2A holoenzyme is a multi-subunit protein complex consisting of an A (structural), B (regulatory), and C (catalytic) subunit. The B subunit is believed to provide substrate specificity. MudPIT analysis was performed by collaborator James Wohlschlegel (UCLA). The results of this analysis identified a single PP2A holoenzyme subunit belonging to the C α subunit. This data confirmed our preliminary data but did not identify the crucial B subunit.

Tasks 4-5 (Aim 1): We then turned our focus to identifying the B subunit of PP2A using molecular techniques. Expression vectors were made that expressed Flag- or HA-tagged B subunits of the B and B' subclasses, which target most known PP2A substrates. These proteins were co-expressed with Myc- or Flag-tagged cyclin E and interactions in vivo determined by immunoprecipitation (IP) Western blot analysis (Task 5). The results of this analysis showed that at least 4 different B subunits, B55 β , B55 δ , B56 β , and B56 δ , strongly interacted with cyclin E1 in vivo (Fig. 1). These results were also confirmed using reciprocal IP reactions. To functionally confirm these B-subunits in regulation of cyclin E stability, we knocked-down expression of these 4 B-subunits in various breast cancer cells lines using siRNA and analyzed the effects on cyclin E protein by Western blotting (Task 4). The results of this analysis showed that knockdown of B55 β or B56 δ had a profound effect on decreasing cyclin E1 levels, as predicted by our central hypothesis (Fig. 2).

Task 6 (Aim 1): To test the importance of PP2A^{B55 β} and PP2A^{B56 δ} in the regulation of cyclin E and potential therapeutic target in breast cancer we knocked-down expression of these B subunits in a panel of breast cancer cell lines. Remarkably, knockdown of either B subunit was found to induce a significant decrease in cyclin E protein levels in most cell lines tested (Fig. 3). The only cell line in which this effect was not observed was Sum149PT, which we previously showed harbors a mutation of the SCF^{Fbxw7} ubiquitin ligase which mediates the ubiquitin-dependent proteolysis of cyclin E1. This data suggested that the effect on cyclin E1 was dependent on a functional degradation system, as predicted by our original hypothesis.

Task 7 (Aim 1): To confirm the effects these B subunits on cyclin E stability, we co-expressed Myc-tagged cyclin E1 with the B-subunits and SCF^{Fbxw7} in HEK293T cells and performed cycloheximide-chase experiments. The results of this analysis showed that co-expression of the B subunit had a remarkable effect on stabilizing cyclin E1, protecting it from degradation by SCF^{Fbxw7} (Fig. 4).

Task 8 (Aim 1): We attempted to confirm the associations of cyclin E with the PP2A B subunits identified above using IP Western blot analysis. This analysis was complicated by the fact that endogenous cyclin E and PP2A B55 β have a molecular weight around 50 kDa, which co-migrates with the IgG heavy chain on SDS-PAGE gels. Furthermore, no commercial antibodies for B55 β and B56 δ have been verified for IP. To

circumvent these problems we elected to express Flag-tagged versions of the B-subunits and attempt to identify their associations with endogenous cyclin E1 protein. This approach successfully verified the interaction between cyclin E1 and the B subunits (Fig. 5).

KEY RESEARCH ACCOMPLISHMENTS:

1. Identification of PP2A^{B55β} and PP2A^{B56δ} in the regulation of cyclin E1 levels and stability in breast cancer cells.
2. Confirmation of PP2A^{B55β} and PP2A^{B56δ} as rational therapeutic targets for decreasing cyclin E levels in breast cancers.

REPORTABLE OUTCOMES:

1. Cloning vectors for expression of Flag- and HA-tagged B55 and B56 class B subunits as well as cyclin E and SCF^{Fbxw7}.
2. Cell lines that stably express B55 and B56 class of PP2A B subunits.
3. Knockdown of PP2A^{B55β} or PP2A^{B56δ} expression in breast cancer cells leads to down-regulation of cyclin E protein.

CONCLUSION: In year 1, we identified the PP2A holoenzyme that interacts and regulates the stability of cyclin E in breast cancer cells. We also showed that knockdown of the expression of PP2A^{B55β} or PP2A^{B56δ} could drive down cyclin E levels in a panel of breast cancer cells lines, including those that overexpress cyclin E1. This data demonstrates that inhibition of these PP2A holoenzymes could be a rational therapeutic strategy for correcting for cyclin E1 overexpression in breast cancer cells. In the coming year, we will further evaluate these holoenzymes as therapeutic targets and implement a chemical library screen to identify small molecule inhibitors of PP2A^{B55β} or PP2A^{B56δ}.

REFERENCES:

1. Sherr, C.J. and Roberts, J.M. (2004). Living with or without cyclins and cyclin-dependent kinases. *Genes Dev* 18, 2699-711.
2. Keyomarsi, K., Tucker, S.L. and Bedrosian, I. (2003). Cyclin E is a more powerful predictor of breast cancer outcome than proliferation. *Nat Med* 9, 152.

APPENDICIES:

None

SUPPORTING DATA:

Figure 1. Associations of cyclin E1 with PP2A subunits B55β and B55δ *in vivo*. HEK293T cells were transfected with plasmids that express Flag-tagged B55 subunits and Myc-cyclin E1. Extracts were then immunoprecipitated with anti-Myc antibodies and association with B55 subunits determined by Western blotting.

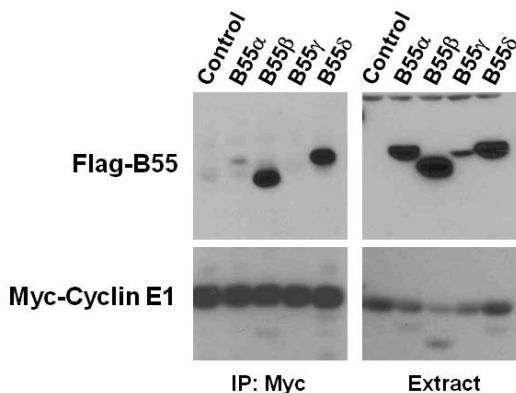


Figure 2. Evaluation of PP2A B-subunit panel on cyclin E levels in breast cancer. MDA-MB-231 cells were transfected with siRNA specific for B-subunits B55 β , B55 δ , B56 β , and B56 δ or control and cyclin E1 levels analyzed by Western blotting. Ku86 is shown as control.

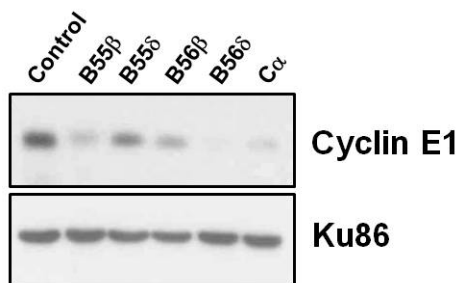


Figure 3. Knockdown of B55 β in a panel of breast cancer cell lines induces down-regulation of cyclin E1 protein levels. The cell lines indicated were transfected with control of B55 β siRNA and the effects on cyclin E1 analyzed by Western blotting. Cell line Sum149PT is known to have a non-functional SCF^{Fbxw7} ubiquitin ligase.

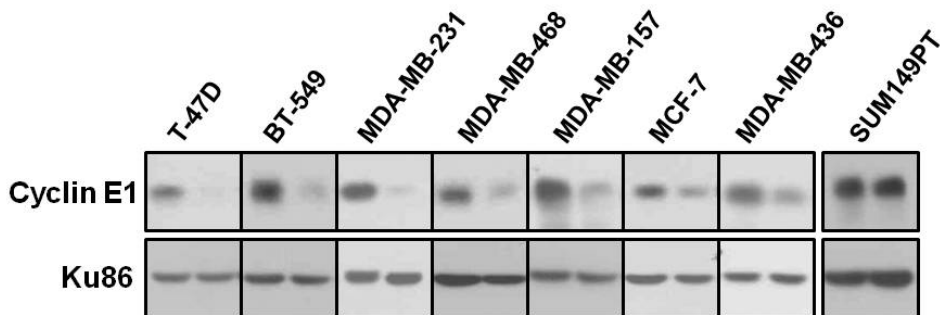


Figure 4. B55 β expression stabilizes cyclin E1 in breast cancer cells. MDA-MB-231 cells were transfected with Myc-cyclin E1 with/without Flag-B55 β and then protein synthesis arrested by treatment with cycloheximide. The effect on cyclin E stability was then determined by Western blot analysis.

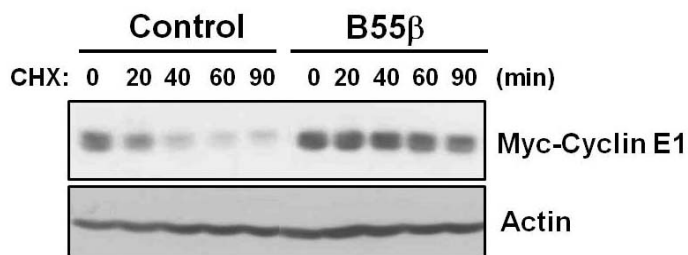


Figure 5. Association of B55 β with endogenous cyclin E. MDA-MB-231 cells were transfected with an expression vector for Flag-B55 β and association with cyclin E1 determined by IP Western blot analysis. 5% extract is shown for reference.

