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TITLE: The Role of the Low Molecular Weight (LMW) Isoforms of Cyclin E in Breast Cancer Tumorigenesis

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14. ABSTRACT Cyclin E is a positive regulator of the G1 to S phase transition of the cell cycle. In complex with CDK2 it is responsible for cells passing the restriction point, committing the cell to a round of DNA replication. Previously this laboratory found that cyclin E is overexpressed and present in lower molecular weight (LMW) isoforms in breast cancer cells and tumor tissues compared to normal cells and tissues. To investigate the role of the LMW forms of cyclin E in tumorigenesis we have developed a model system of non-tumorigenic breast cells overexpressing the individual isoforms of cyclin E. Using this model system we have determined that the LMW forms of cyclin E cause increased proliferation of non-tumorigenic breast epithelial cells. This proliferation can be inhibited by preventing the processing of the LMW forms of cyclin E through the expression of elafin in these cells.					
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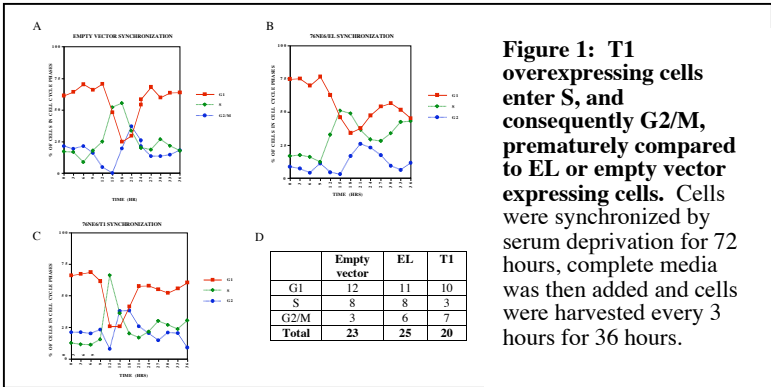
Introduction:

The frequent appearance of the low molecular weight (LMW) forms of cyclin E and their correlation with poor prognosis in breast cancer patients indicate that the LMW forms may be oncogenic, playing specific roles in the development of malignancies. The purpose of this application is to determine the role of the LMW forms of cyclin E in the transformation of normal cells into neoplastic cells. The outcome of this research will be significant in that it will delineate a novel mode of deregulation of cyclin E and possibly identify a new oncogene involved in breast cancer tumorigenesis therefore providing the rationale for novel drug design.

Body:

To determine the role of the low molecular weight (LMW) isoforms of cyclin E in tumorigenesis, we are generating a model system of immortalized, non-tumorigenic mammary epithelial (76NE6) cells overexpressing each of the cyclin E isoforms. We will then characterize each of the clones overexpressing the individual isoforms and compare these cells to the untransfected and vector alone transfected cells. Despite numerous attempts, utilizing several methods, we have not been able to generate a stable clone expressing the lowest molecular weight isoform (T2). Therefore, for our first aim we will be comparing stable clones of 76NE6 cells overexpressing the full length cyclin E isoform (EL) to cells overexpressing the low molecular weight isoform (T1) and the empty vector (pcDNA 4.0) as a negative control.

Our first aim is to generate and characterize stable breast cell lines overexpressing the LMW forms of cyclin E. We have shown that the LMW isoforms of cyclin E have increased kinase activity compared to the full length cyclin E. This activity translates in to a shortened S phase of the cell cycle and increased colony formation. We had previously shown the cell cycle differences using growth curves and cell cycle analysis of asynchronous cells. We have further confirmed our findings by synchronizing each of the stable cell lines by serum deprivation and harvesting cells for cell cycle analysis and westerns every 3 hours for a 36-hour time course (figure 1).



In our second aim we are examining the tumorigenic potential of non-tumorigenic cells overexpressing the cyclin E isoforms. Hanahan and Weinberg summarized eight characteristics of tumor cells [1]. We have chosen assays that serve as surrogate indicators of these characteristics to determine the tumorigenic phenotype of our cell lines. For example, tumor cells acquire self-sufficiency in growth signals. In

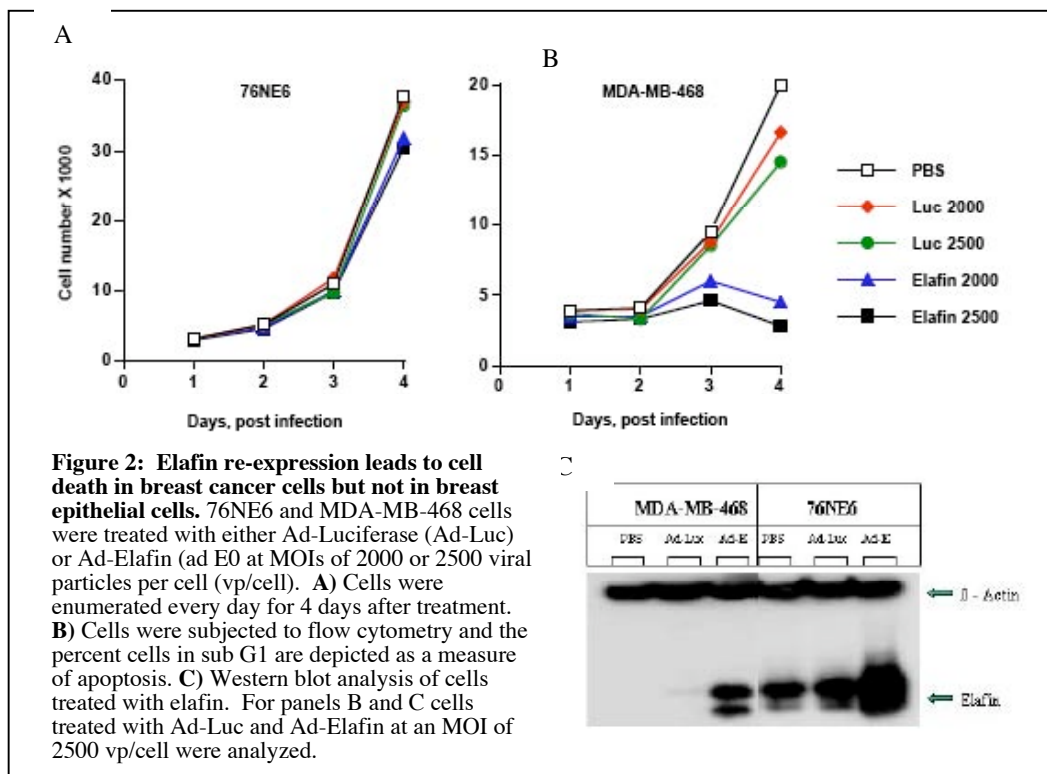
culture, normal cells require a proper substratum for signaling components to activate their growth signals. Tumor cells do not depend on attachment to a substratum for this signaling to occur. Therefore we have tested the ability of each of our cyclin E isoform overexpressing cell lines to grow in soft agar. Our results show that the low molecular weight isoforms do not provide cells the machinery to grow in the absence of anchorage. Another characteristic of tumor cells is their ability to invade tissue and to metastasize. This is an extremely detrimental phenotype of tumor cells as it is the metastasis that is lethal to the patient, not the primary tumor. To determine whether the LMW isoforms of cyclin E provide non-tumorigenic cells the ability to invade a matrix, we tested the ability of our stable cell lines to invade a matrigel membrane (table 1). Our results show that the LMW forms of cyclin E do not give 76NE6 cells an increased potential to invade a matrix membrane. However, in our model system, the full-length cyclin E is endogenously expressed alongside our exogenously expressed isoforms. Therefore, in our T1 and T2 cell lines we are concerned that despite being overexpressed, the effects of the LMW isoforms of cyclin E could be neutralized by the expression of endogenous, full-length cyclin E. We have generated a vector that will express shRNA against the 3'UTR region of cyclin E to silence the endogenous cyclin E, but not the exogenous cyclin E. These vectors are currently being expanded in bacterial cultures for

transfection in to our cell lines.

Cell line	# of invading cells
MDA-MB-436	37
Empty vector (clone)	18
EL (clone)	19
T1 (clone)	21
T2 (pool)	23
MCF-7	0

Table 1: The LMW forms of cyclin E do provide cells an increased potential to invade a membrane. 25,000 cells from each of our 76NE6 stable cell lines (empty vector, EL, T1), a pool of cells overexpressing T2, MDA-MB-436 cells (positive control) and MCF-7 cells (negative control) were plated on a matrigel matrix in a chamber slide. After 24 hours, the slides were analyzed microscopically and cells in the lower compartment that had crossed the matrigel membrane were counted.

We have shown that the LMW forms of cyclin E deregulate the cell cycle of non-tumorigenic cells [2], cause resistance to inhibition by p21 and p27[3], and have been correlated with a poor prognosis in breast cancer patients [4] the LMW forms of cyclin E could provide a therapeutic target for breast cancer. Elafin is an endogenously expressed protein that inhibits elastase. Increased levels of elastase have been shown to be a strong prognostic indicator in breast cancer patients, associated with recurrence and death. Elastase has also been shown to generate tumor-specific low molecular weight forms of cyclin E[5]. To directly show that elafin can inhibit the proliferation of tumor cells overexpressing the LMW forms of cyclin E, we used a recombinant adenovirus to overexpress elafin in normal and tumor cells. We found that elafin caused a complete inhibition of cell proliferation in the invasive tumor cells overexpressing all the LMW forms of cyclin E (MDA-MB-468) and not the immortalized mammary epithelial cells which express only the full-length cyclin E (76NE6) (Figure 2A). The results show that while the proliferation of 76NE6 cells was unaffected by any of the viruses used, the proliferation of MDA-MB-468 breast cancer cells was completely inhibited by Ad-Elafin. Flow cytometry of the treated cells showed that only tumor cells treated with Ad-Elafin accumulated in sub-G1, indicative of apoptotic cell death. Normal cells were completely unaffected by elafin overexpression (Figure 2B). Western blot analysis shows that elafin was expressed at high levels in both the tumor cells and breast epithelial cells (Figure 2C). The introduction of Ad-Elafin resulted in overexpression of elafin in the MDA-MB-468 cell line which appears to be closer to physiological levels since the viral induced expression was similar to the amount of elafin expression seen in the untreated 76NE6 breast epithelial cells (i.e. compare lane 3 to lane 4 in Figure 2C). Furthermore, Ad-Elafin treatment resulted in overexpression of elafin to supraphysiologic levels in the 76NE6 breast epithelial cell line without any impact on proliferation.



Key Research Accomplishments:

- Confirmed proliferative characteristics of 7NE6 cells overexpressing the LMW isoforms of cyclin E by synchronizing the cells and analyzing the changes in duration of cell cycle phases
- Determined the ability of 76NE6 cells overexpressing the LMW isoforms of cyclin E to grow in anchorage independent conditions
- Determined the ability of 76NE6 cells overexpressing the LMW isoforms of cyclin E to invade a matrix membrane
- Introduced adenoviral elafin into tumor (MDA-MB-468) cells and non-tumorigenic (76NE6) cells to inhibit LMW cyclin E expression and as a result the proliferation of the tumor cells.

Reportable Outcomes

- Presented data at Baylor College of Medicine's Breast Disease Research Group Seminar Series
- Presented poster at the Cold Spring Harbor- Cell Cycle meeting
- Presented poster at the University of Texas-M.D. Anderson Cancer Center Trainee Recognition Day
- The cell lines generated in this proposal (76NE6/empty vector, 76NE6/EL and 76NE6/T1) have been given to other investigators to study the specificity of CDK inhibitor drugs.

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Conclusions:

- The LMW forms of cyclin E cause increased proliferation of non-tumorigenic, breast epithelial cells
- The LMW isoforms of cyclin E do not appear to render the cells independent of extracellular growth signals as determined by soft agar colony formation assays
- The LMW isoforms of cyclin E do not appear to play a role in metastasis of the non-tumorigenic breast epithelial cell lines, as the cyclin E isoforms did not increase the ability of the cells to invade a matrix membrane
- Because the LMW isoforms cause increased proliferation and genomic instability, an underlying characteristic of cancer, and are correlated with poor prognosis, the question exists whether these LMW isoforms of cyclin E serve as a potential target for breast cancer therapy
- Our studies using expression of elafin show that with increased elafin, the generation of the LMW forms is inhibited as is the proliferation of tumor cells
- **Therefore, the LMW forms of cyclin E play a role in the increased proliferation of breast epithelial cells and this can be reversed by the expression of elafin**

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