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Award Number: DAMD17-03-1-0675

TITLE: The Role of Calgranulin Overexpression in Breast Cancer Progression

PRINCIPAL INVESTIGATOR: John J. Pink, Ph.D.

CONTRACTING ORGANIZATION: Case Western Reserve University
Cleveland, Ohio 44106

REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2004	Annual (15 Aug 03 - 14 Aug 04)

4. TITLE AND SUBTITLE
The Role of Calgranulin Overexpression in Breast Cancer Progression

5. FUNDING NUMBERS
DAMD17-03-1-0675

6. AUTHOR(S)
John J. Pink, Ph.D

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Case Western Reserve University
Cleveland, Ohio 44106

E-Mail: jrp16@cwru.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The mechanism responsible for the progression of breast cancer to estrogen independence is currently unknown. We have demonstrated a strong correlation between elevated expression of Calgranulin A and Calgranulin B and the loss of estrogen dependence. Experiments using estrogens and antiestrogens have demonstrated that this is not the result of a direct interference with estrogen receptor signaling and must therefore require additional steps. Interestingly, we have also shown that expression of calgranulins in estrogen independent breast cancer cells is quite variable and may be differentially regulated during the cell cycle. We are currently in the process of determining if forced overexpression of calgranulin A and/or calgranulin B can drive cells to lose their dependence on estrogens for growth

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, Antiestrogens, Calgranulins, Progression	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

The progression of breast cancer from a state of estrogen dependence to estrogen independence is a critical development in the clinical history of this disease. To date, little is known about the crucial changes that lead to the appearance of this more aggressive tumor phenotype which is of importance clinically because it results in resistance to the antiproliferative effects of selective estrogen response modifiers (SERMs), most notably tamoxifen (1-3). In an attempt to understand the changes that allow breast cancer cells to lose estrogen dependence and therefore become resistant to SERMs (4-6), we performed an experiment to identify gene transcripts that were significantly elevated in two unrelated breast cancer cell lines (T47D:C4:2W & MCF-7:2A) that were selected for growth in estrogen-free media, as compared with their estrogen-dependent progenitor cell lines (T47D:A18 & MCF-7:WS8). Using microarray analysis with the Affymetrix U95A gene chip, we identified a number of genes that were greatly elevated in both resistant lines, when compared with their sensitive parental lines. The analysis was designed to focus only on transcripts that were elevated in both lines in order to exclude transcripts that were either spuriously elevated or whose increase was restricted to a single cell line. This was intended to increase the chances of finding genes that were involved in common pathways leading to estrogen independence. Most interestingly, in both pairs of cell lines the two most highly elevated mRNAs were for calgranulin A and its dimerization partner, calgranulin B (7, 8). The finding that the transcripts for two related gene products were elevated more than 30 fold in the case of calgranulin B and more than 10 fold for calgranulin A, in two independently derived cell lines, strongly suggested a mechanistic pathway that may be responsible for the loss of estrogen dependence in these breast cancer cell lines. This may represent a widespread mechanism by which cells can progress to a more aggressive phenotype accompanied by a loss of estrogen dependence.

Body

Experiments were undertaken to demonstrate a correlation between calgranulin expression and lack of estrogen dependence in breast cancer cell lines. A panel of breast cancer cells, including lines derived from common parental lines (e.g. [MCF-7:WS8, MCF-7:2A and MCF-7:C4-12-5]; [T47D:A18 and T47D:C4:2W]) as well as unrelated breast cancer lines (MDA-MB-231, ZR-75-1 and MDA-MB-468) were run in a standard western blot and probed for calgranulin A (Cal A) using the 85C2 monoclonal antibody. The blot was then reprobed with an antibody to estrogen receptor alpha (ER-α). The blot was finally probed with α-tubulin to confirm equal loading.

The data in Figure 1 and Table 1 show a significant correlation between loss of estrogen dependence and expression of Cal A. This suggests that expression of Cal A may be either an indicator of the propensity of cells to lose estrogen dependence or a result of the loss of estrogen dependence. The only cell line that does not fit this model is the MDA-231 cell line, which expresses neither ER-α nor Cal A.

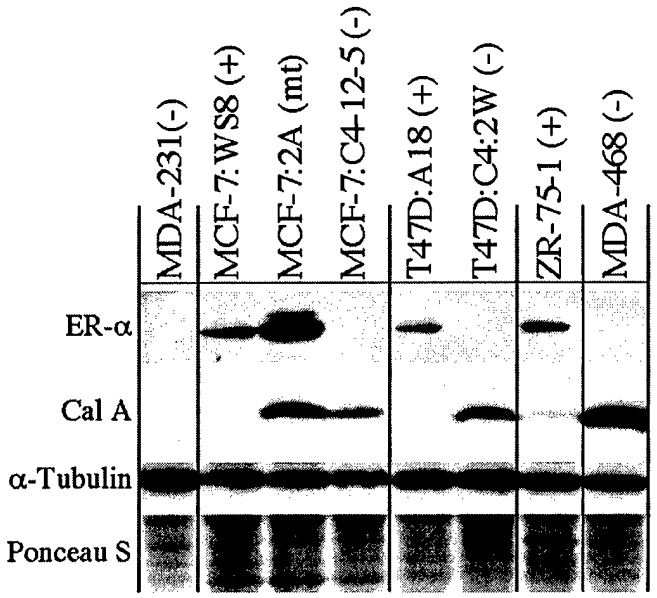

Figure 1: Expression of calgranulin A in breast cancer cell lines. Whole cell extracts were prepared from log phase cells and run in a standard western blot. The blot was first probed with a monoclonal antibody to Calgranulin A (Cal A), reprobed with an antibody to estrogen receptor alpha (ER-α) and finally probed with an antibody to α-tubulin as a loading control. Ponceau S staining of the membranes is included as a second control for loading.

Confocal microscopy was used to visualize Cal A expression in a number of the cell lines used in these studies. Interestingly the expression of Cal A was not uniform throughout the culture. The range of expression within each expressing cell line was quite wide. A representative micrograph for MCF-7:2A is shown in figure 3. Further analysis of these phenomena will be carried out in the upcoming year. This could indicate that expression of calgranulins is cell cycle regulated. Experiments to address this possibility are in progress.

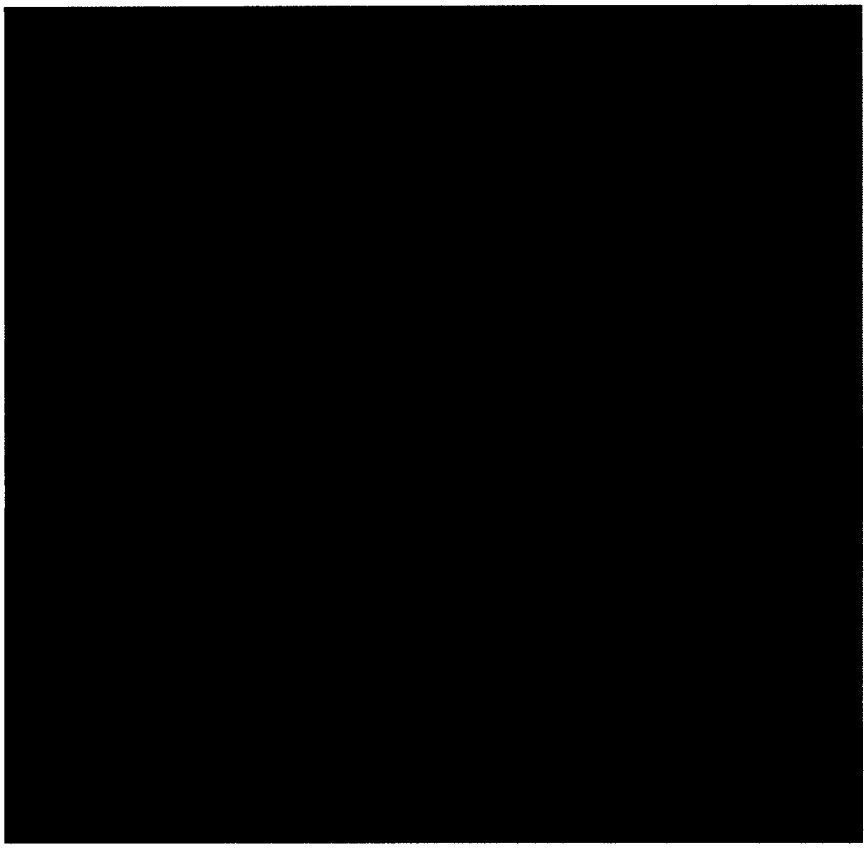

Figure 2: Expression of calgranulin A in MCF-7:2A cells. Log phase cells were grown on coverslips and fixed with formaldehyde. Cells were then incubated with a mouse monoclonal antibody to Calgranulin A, followed by a FITC-labeled anti-mouse secondary antibody. Calgranulin A expressing cells were then visualized using confocal microscopy.

To generate cell lines which mimic the Cal A expression in the MCF-7 and T47D background, MCF-7WS8 and T47D:A18 cells were transfected with a construct received from Karen Ross (Univ of Minnesota) which included the cDNAs for Cal A and Cal B separated by an IRES . Pooled populations were isolated and screened for Cal A and Cal B expression (see Figure 3). Cal A expression was reasonable, however, Cal B expression was quite low. It was subsequently revealed by the manufacturer of the vector (Clontech Biotech) that the IRES included in the original construct used to generate this vector is attenuated and regularly gives rise to low or undetectable expression of the downstream cDNA. Unfortunately this information was forwarded only after the cell lines were generated and initially screened. This is consistent with our observations and therefore these cells lines are of limited use in these studies. To rectify this problem I have contacted another lab (Eckert and Broome, CWRU) and am in the process of using new vectors that encode Cal A and Cal B on separate plasmids. These will be transfected into the MCF-7:WS8 and T47D:A18 cell either separately or together.

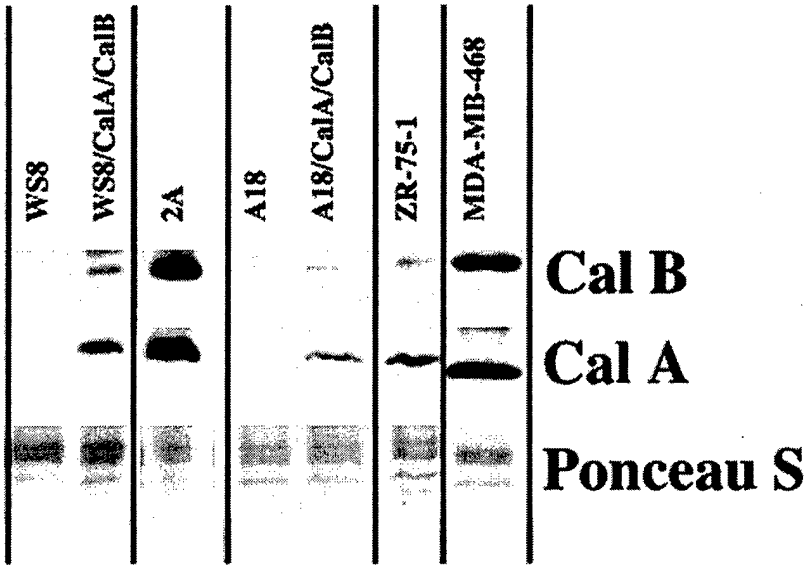

Figure 3: Expression of Cal A and Cal B after transfection. MCF-7:WS8 (WS8) and T47D:A18 (A18) were transfected with an expression plasmid containing cDNAs for Cal A and Cal B separated by an internal ribosome entry site (IRES). Populations of cells were screened for Cal A and Cal B expression using a standard western blot as described for Figure 1

To address the possibility that Cal A expression may be a direct result of inhibition of ER-α signaling, WS8 and A18 cells were grown in estrogen free media for 4 days to remove all endogenous steroids, replated and then treated with 1nM 17ß-estradiol (pure estrogen), 100nM 4-hydroxytamoxifen (SERM) or 100nM ICI 182,780 (pure antiestrogen) for an additional 2 days. Whole cells lysates were then prepared and run in a standard western blot. ER-α expression serves as a control for the effects of the steroids used. Estradiol has been shown to cause a downregulation of ER-α, 4-OHT has been shown to stabilize ER-α and ICI 182,780 has been shown to cause a rapid degradation of ER-α. The response is similar in T47D cells with the exception that estradiol does not cause a downregulation of ER-α (6). In both the WS8 and A18 cell lines, Cal A remained below detectable levels in all treatment groups. Control lysates from MCF-7:C4-12-5 and T47DC4:2W cells were included as positive controls for Cal A. This confirms that Cal A expression is not likely to be a direct downstream target of estrogen receptor signaling and probably requires other pathways to become overexpressed.

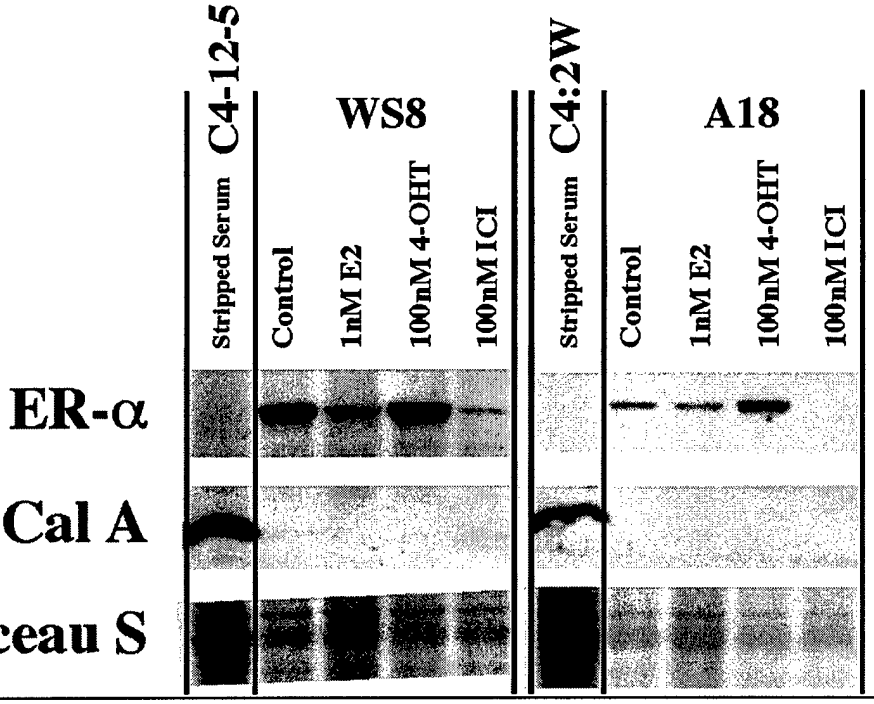

Figure 4: Estrogen/antiestrogen regulation of Cal A expression. MCF-7:WS8 and T47D:A18 cells were grown in estrogen-free media for 4 days, replated and treated with the indicated drugs for an additional 2 days. Whole cell extracts were prepared and run in a standard western. Cal A and ER-α expression were determined as described above.

Table 1: Cell Line Characteristics

Cell Line	ER Status	Estrogen dependence	Cal A Expression
MDA-231	-	-	-
MCF-7:WS8	++++	+++	-
MCF-7:2A	++++(mutant)	-	+++
MCF-7:C4-12-5	-	-	++
T47D:A18	+++	++++	-
T47D:C4:2W	-	-	+++
ZR-75-1	+++	+++	+
MDA-468	-	-	++++

Key Research Accomplishments

- Established correlation between estrogen independence and calgranulin overexpression in established breast cancer cell lines
- Initial observation of differential calgranulin expression in log phase cells
- Generated shRNA vector specific for calgranulin A

Reportable Outcomes

Preliminary data generated from this award were included in 3 grant proposals, an Idea Award from the DOD, and RO1 to the NIH and a Basic/Translational Grant to the Komen Foundation.

MCF-7 and T47D cell lines expressing a bicistronic Cal A and Cal B vector have been generated; however expression of Cal B is very low, making their usefulness questionable.

Conclusions

These studies are continuing with a no cost extension. During the extension the studies described in the statement of work will be completed, with the intent of describing the role of calgranulin overexpression in breast cancer progression, primarily focused on progression to estrogen independence. The most important work will be focused upon generating the cells lines expressing Cal A and Cal B and the investigation of the role these proteins play in the development of estrogen independence in breast cancer cell lines.

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