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Award Number: W81XWH-06-1-0478

TITLE: Eosinophil Granular Protein(s) Modulate Tumor Metastasis  
Marker Gene Expression

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Washington, DC 20060

REPORT DATE: May 2008

TYPE OF REPORT: Annual Report

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

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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                             |                              |                                                   | Form Approved<br>OMB No. 0704-0188                        |                                                              |
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| 1. REPORT DATE (DD-MM-YYYY)<br>16-05-2008                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |                             | 2. REPORT TYPE<br>Annual     |                                                   | 3. DATES COVERED (From - To)<br>17 APR 2007 - 16 APR 2008 |                                                              |
| 4. TITLE AND SUBTITLE<br>Eosinophil Granular Protein(s) Modulate Tumor Metastasis<br>Marker Gene Expression                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |                             |                              |                                                   | 5a. CONTRACT NUMBER<br>031012-1381                        |                                                              |
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| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>AND ADDRESS(ES)<br>Howard University<br>2041 Georgia Ave., NW, #530<br>Washington, DC 20060                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |                             |                              |                                                   | 8. PERFORMING ORGANIZATION REPORT<br>NUMBER               |                                                              |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>US Army Medical Research and<br>Materiel Command<br>Fort Detrick, Maryland 21702-<br>5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |                             |                              |                                                   | 10. SPONSOR/MONITOR'S ACRONYM(S)                          |                                                              |
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| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br><br>Approved for public release; Distribution limited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |                             |                              |                                                   |                                                           |                                                              |
| 13. SUPPLEMENTARY NOTES                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |                             |                              |                                                   |                                                           |                                                              |
| 14. ABSTRACT - MCF-7 breast cancer multicellular tumor spheroids have been used as the model system for evaluating the effect of eosinophil granular proteins as well as pro-/anti-inflammatory cytokines on tumor growth and proliferation. More specifically this study attempts to evaluate the effect of these proteins on the expression of oncogenes which modulate growth and proliferation of tumors. These oncogenes include erbB2/Her-2-neu, cyclin D1, cyclin E1, p21, p27, p53, e-cadherin and n-cadherin. Eosinophil cell lines established from an allergic asthmatic individual were batch cultured and used to isolate eosinophil granular proteins (MBP, EPO and ECP). Additional protein isolation efforts include perforin and granzyme b which are also produced by eosinophils and which are known to be a mechanism by which cytotoxic T cells and natural killer cells kill tumor targets. SDS-PAGE analysis of 18 and 21 HCl extractions from eosinophil granules (spontaneously released in culture supernatants and cellular extracts, respectively) revealed molecular weight bands for MBP, EPO, and ECP in 14 of the supernatant granule extracts and in 20 of the 21 cellular granule extracts. MCF-7 spheroids were treated with 10-fold increasing suboptimum doses of MBP and cytokines IL-4, TNF-alpha, IL-10 and IL-12 individually for 24, 48 and 72 hrs. IL-4, MBP and IL-12 downregulated expression of all genes except erbB2; TNF-alpha downregulated erbB2 (5ng), CyD1, CyE1, p21, p27 and p53, while IL-10 downregulated CyE1, p21, p53 and e-cadherin expression. The data strongly suggest the potential anti-cancer effects of MBP at very low doses. The possible collaborative effect of MBP, other granular proteins and the cytokines studied with known anti-cancer drugs to effectuate greater efficacy of anti-cancer drug activity may be the direction for these studies. |                             |                              |                                                   |                                                           |                                                              |
| 15. SUBJECT TERMS none listed.                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                             |                              |                                                   |                                                           |                                                              |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |                             |                              | 17. LIMITATION<br>OF ABSTRACT<br><br>Unclassified | 18. NUMBER<br>OF PAGES<br><br>50                          | 19a. NAME OF RESPONSIBLE PERSON<br>Paulette Furbert-Harris   |
| a. REPORT<br>Unclassified                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | b. ABSTRACT<br>Unclassified | c. THIS PAGE<br>Unclassified |                                                   |                                                           | 19b. TELEPHONE NUMBER (include area<br>code)<br>203-806-7722 |

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### 3. Introduction

Immunological therapeutic strategies for breast cancer and other cancers have not had great success in the past, however new efforts with cancer vaccines, and gene therapy are currently being examined (1-5). In our laboratory we examine the eosinophil as an anti-cancer effector cell. *In vitro* studies with activated eosinophils and breast cancer cell lines demonstrated binding of eosinophils to tumor cells, followed by growth inhibition (6). Moreover, infiltrated eosinophils released their granular contents into MCF-7 multi cellular tumor spheroids (7). Eosinophil granular contents include granular proteins major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) (8). Additional granular proteins include granzyme b and many cytokines (9, 10-13). In this investigation we will isolate eosinophil granular proteins from eosinophil cell lines and evaluate their effects on the expression of oncogenes involved in growth and proliferation of tumors, erbB2, cyclin D1, cyclin E1, p21, p27, p53 and adhesion molecules e-cadherin and n-cadherin. We will also examine the effect of proinflammatory and inflammatory cytokines on oncogene expression.

### 4. Body

***Isolation of Granular Proteins from Eosinophils.*** Cell lines GRC.014.22 and GRC.014.24 (combined) and also SD.031.22(individual) were expanded in culture for 1 week, after which they were counted and seeded into T162 tissue culture flasks at  $1 \times 10^6$  cells/ml (100 mls per flask). The flasks were incubated at 37C, 5% CO<sub>2</sub> atmosphere for 72 hrs or until the culture media was spent. The cells were harvested and counted. The supernatants were pooled and stored at -70C for future isolation of eosinophil granules. Eosinophils ( $1.8 \times 10^9$ ) were pelleted, then washed with 0.34M sucrose without heparin, then centrifuged at 200-250g. for 8 minutes. Cells were equally distributed into three tubes in 20 ml of 0.34M sucrose with heparin. The cells were pipetted 10-15 times over 2 minutes at room temperature, then pooled. An additional 300 ml of 0.34M sucrose with heparin was added. The viscous suspension was then forced through an 18 gauge needle, after which 200 ml of 0.34M sucrose without heparin was added. The mixture was stored at 4C overnight. The granular suspension was centrifuged at 400g for 10 min at 4C. The supernatant – containing crude granular proteins were centrifuged at 400g for 10 min to collect more granules. The combined granular pellet was rinsed gently with cold 1X PBS, then re-suspended in cold 0.01N HCL, incubated for 10 minutes and then centrifuged at 25000g for 20 minutes at 4C. Supernatants were collected, stored at -70C and treatment with 0.01N HCL was repeated (10-15X) on the cellular pellet. Granular protein lysates were concentrated using amicon filter. Concentration of protein isolated was determined using Biorad Experion Protein Quantitation Kit. The stored culture supernatants were thawed and centrifuged first at 250g to remove any cells and cellular debris, then centrifuged at 400g for 10 min. The pooled granular pellet was treated as described above with 0.01N HCl 18 different times, collecting and storing 18 individual fractions.

***Resolution of Granular Protein lysates by Polyacrylamide Gel Electrophoresis.***

Granular protein lysates, crude and amicon-filtered (5 kD) were separated by gel electrophoresis' using Ready Gel precast gels (Bio-Rad), 15% Tris-HCl. This percentage gel allows for protein separation ranging from 10 kD to 200 kD. Our proteins of interest range in size from 14 kD to 75 kD.

***Western Blots.*** Will be performed to confirm presence of specific proteins of interest to the study.

***Eosinophil cell lines.*** GRC.014.22-hypodense cells from an individual with mild asthma; GRC.014.24- hyperdense cells from the same individual and SD.031.22-hypodense cells from an individual with both asthma and breast cancer, were used to isolate granules.

***MCF-7 Multicellular Tumor Spheroids.*** Of the 4 cell lines evaluated MCF-7 breast tumor cells formed the best spheroids'. When 200 up of cells ( $5 \times 10^6$  cells/ml) were seeded into 96-well round bottom plates percolated with a film of 1% agarose, large spheroids were formed ( $> 1500$  um) with well-defined necrotic cores within 4 days. These spheroids were treated with suboptimum concentrations of MBP and cytokines. Although these concentrations may affect the growth of the spheroids, these spheroids remained intact such that RNA could be isolated. Each 96-well plate (96 spheroids) was used per treatment.

***Real Time/Gene Expression Assay.*** cDNA for each RNA sample was obtained using the Bio-Rad iScript Reverse Transcription Kit. Real-time PCR was set-up for each treatment to examine the effect of eosinophil granular protein MBP and cytokines TNF $\alpha$ , IL-4, IL-10, and IL-12) on MCF-7 MTS oncogene expression of the following markers erbB2, cyclin D1, cyclin E, cyclin kinase inhibitors, p21 and p27, p53, E-cadherin and N-cadherin.

## **5. Key Research Accomplishments**

- Isolation of eosinophil granular proteins from hypodense and hyperdense eosinophil cell lines.
- Treatment of MCF-7 multicellular tumor spheroids (MTS) with MBP and proinflammatory and noninflammatory cytokines, IL-4, TNF $\alpha$ , IL-10 and IL-12.
- Isolation of RNA from spheroids treated with MBP and cytokines, 3doses each for 24, 48 and 72 hrs.
- Oncogene expression in multicellular tumor spheroids treated with MBP, IL-4, TNF $\alpha$ , IL-10 and IL-12

## **6. Reportable Outcomes**

- Chair, Ph.D. Dissertation Committee
- Member, Ph.D. Dissertation Committee
- Mentor, 3 Ph.D students
- Chair, Internal Advisory Committee, Research Centers in Minority Institute (RCMI) Program

- Abstract, Era of Hope Breast Cancer Research Program: Eosinophil Granular Protein Cocktail Modulates the Expression of Cancer Growth and Metastasis Genes in MCF-7 Multicellular Spheroids (June, 2008)
- **Manuscript** – Robbins CM, Hernandez W, Ahaghotu C, Bennett J, Hoke G, Mason T, Pettaway CA, Vijayakumar S, Weinwrich S, **Furbert-Harris P**, Dunston GM, Powell IJ, Carpten JD, Kittles RA. (2008) The Prostate
- Invitee, Eighth International Conference of AntiCancer Research, Kos, Greece.

## 7. Discussion/Conclusion

The overall objective of the study is to determine the effect of eosinophil granular protein MBP and other granular proteins (ECP, EDN) alone and in combination with proinflammatory and noninflammatory cytokines on the expression of cancer genes involved in regulating growth and proliferation, resistance to apoptosis, invasion and metastasis. The tasks of the second phase were :

- a. Batch culture eosinophil cell lines for eosinophil granular protein isolation. Isolate proteins from individual cell line groups (hypodense cells from asthmatic and cancer/asthma and nonasthma/noncancer individuals) to compare their effects on tumor growth.
- b. Dose response and time kinetic studies of spheroids treated with eosinophil protein extract, purified MBP, IL-4, IL-10, IL-12, TNF $\alpha$  and combinations. RNA isolations from whole spheroids and spheroid fractions of proliferative and quiescent cells. Quantitate and store RNA samples.
- c. Detection of cell adhesion molecules (ICAM, VCAM-1) by immunohistochemistry.
- d. Western blot and apoptosis studies post MBP and cytokine treatment.

In deciding to prepare an abstract for the Era of Hope meeting, held at the Baltimore Convention Center, June 25 – 28, 2008, I decided to switch tasks and determine oncogene expression of whole spheroids treated with the various agents, during this period. Immunohistochemistry and western blot analyses will be performed in phase III. In the 18-21 granule protein extracts (supernatant and cellular), MBP, ECP, EPO, could be found as far out as fraction 14 in the supernatant granules while with the cellular granules they were detected as far out as fraction 21. These data are similar to that described by Gleich et al who performed 31 extractions on cellular granules (14).

Failure to detect these proteins in all fractions could be due to low protein concentration in these fractions.

We have isolated granules from a second culture preparation and will carry out 31 extractions with both supernatant as well as cellular granules. With regards to the oncogene expression, although suboptimum concentrations were used the treatment doses and times resulted in down regulation of the oncogenes that cause unrestricted growth and proliferation of tumors cells. As early as 24 hrs TNF $\alpha$  (low and high dose) completely downregulated p21, p27, and p53, with p21 being downregulated throughout the incubation period (24-72hr). Similarly IL-4 as early as 24 hrs (low and high dose) down regulated the expression of all the oncogenes tested.

Although this effect was reversed at 48 hrs, at 72 hrs again the expression of CyD1, CyE1 p21 (low dose) and E-Cadherin were down regulated thus suggesting a biphasic reaction. This biphasic response was also observed with IL-12 and MBP (72 hrs low dose downregulated expression all genes while IL-10 was more variable in its effects.

In summary, eosinophil granular protein, MBP as well as pro and anti-inflammatory cytokines, IL-4, TNF $\alpha$ , IL-10 and IL-12 which are known to be preformed in eosinophil granules, can downregulate oncogene expression. The analyses of expression of these genes in the various proliferative zones of the spheroid will be determined in year 3 as well as the detection of adhesion molecules post treatment.

## 8. References

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## 9. Appendices

Figure 1. Eosinophil Cell Line.

Figure 1. Legend

Figure 2. SDS-PAGE Profile of Granular Protein Extracts  
(Supernatant Granules)

Figure 2. Legend

Figure 3. SDS-PAGE Profile of Granular Extracts

Figure 3. Legend

Figure 4. SDS-PAGE Profile of Eosinophil Granular Proteins  
Isolated from Granules Released in Eosinophil Cultured Supernatants

Figure 4. Legend

Figure 5. Composite SDS-PAGE Profile of Eosinophil Granular Proteins  
Isolated from Cultured Eosinophil Cell Lines

Figure 5. Legend

Figure 6. Effect of TNF-alpha on Oncogene Expression in MCF-7 MTS (24 hrs)

Figure 6. Legend

Figure 7. Effect of TNF-alpha on Oncogene Expression in MCF-7 MTS (48 hrs)

Figure 7. Legend

Figure 8. Effect of TNF-alpha on Oncogene Expression in MCF-MTS (72 hrs)

Figure 8. Legend

Figure 9. Effect of IL-4 on Oncogene Expression in MCF-7 MTS (24 hrs)

Figure 9. Legend

Figure 10. Effect of IL-4 on Oncogene Expression in MCF-7 MTS (48 hrs)

Figure 10. Legend

Figure 11. Effect of IL-4 on Oncogene Expression in MCF-7 MTS (72 hrs)

Figure 11. Legend

Figure 12. Effect of IL-10 on Oncogene Expression in MCF-7 MTS (24 hrs)

Figure 12. Legend

Figure 13. Effect of IL-10 on Oncogene Expression in MCF-7 MTS (48 hrs)

Figure 13. Legend

Figure 14. Effect of IL-10 on Oncogene Expression in MCF-7 MTS (72 hrs)

Figure 14. Legend

Figure 15. Effect of IL-12 on Oncogene Expression in MCF-7 MTS (24 hrs)

Figure 15. Legend

Figure 16. Effect of IL-12 on Oncogene Expression in MCF-7 MTS (48 hrs)

Figure 16. Legend

Figure 17. Effect of IL-12 on Oncogene Expression in MCF-7 MTS (72 hrs)

Figure 17. Legend

Figure 18. Effect of MBP on Oncogene Expression in MCF-7 MTS (24 hrs)

Figure 18. Legend

Figure 19. Effect of MBP on Oncogene Expression in MCF-7 MTS (48 hrs)

Figure 19. Legend

Figure 20. Effect of MBP on Oncogene Expression in MCF-7 MTS (72 hrs)

Figure 20. Legend

## SUPPORTING DATA

**Figure 1. Eosinophil Cell Line**



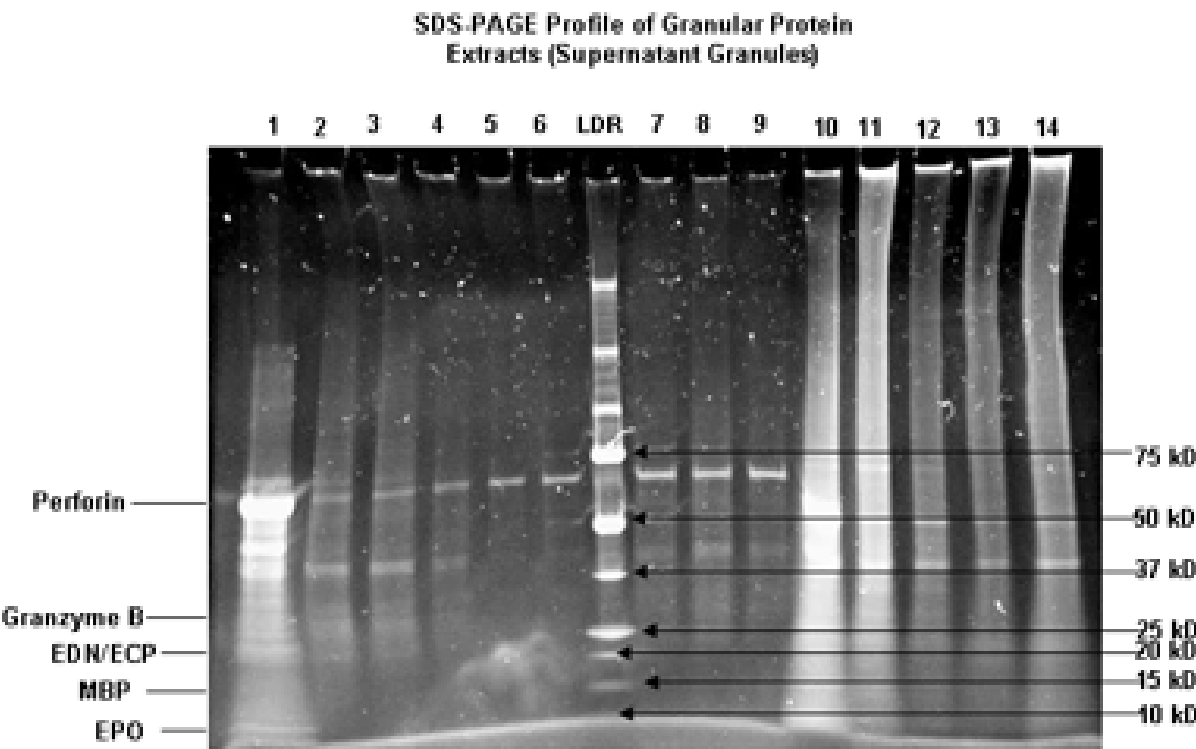
**Eosinophil Cell line at  
Initial Seeding**

**Eosinophil Cultures After  
4 Days of Culturing**

GRC.014.22; GRC.014.24  
BJA.060.22; BJA.060.24  
CTA.052.22; CTA.052.24  
LAE.013.22; LAE.013.24  
SD.031.22; SD.031.24

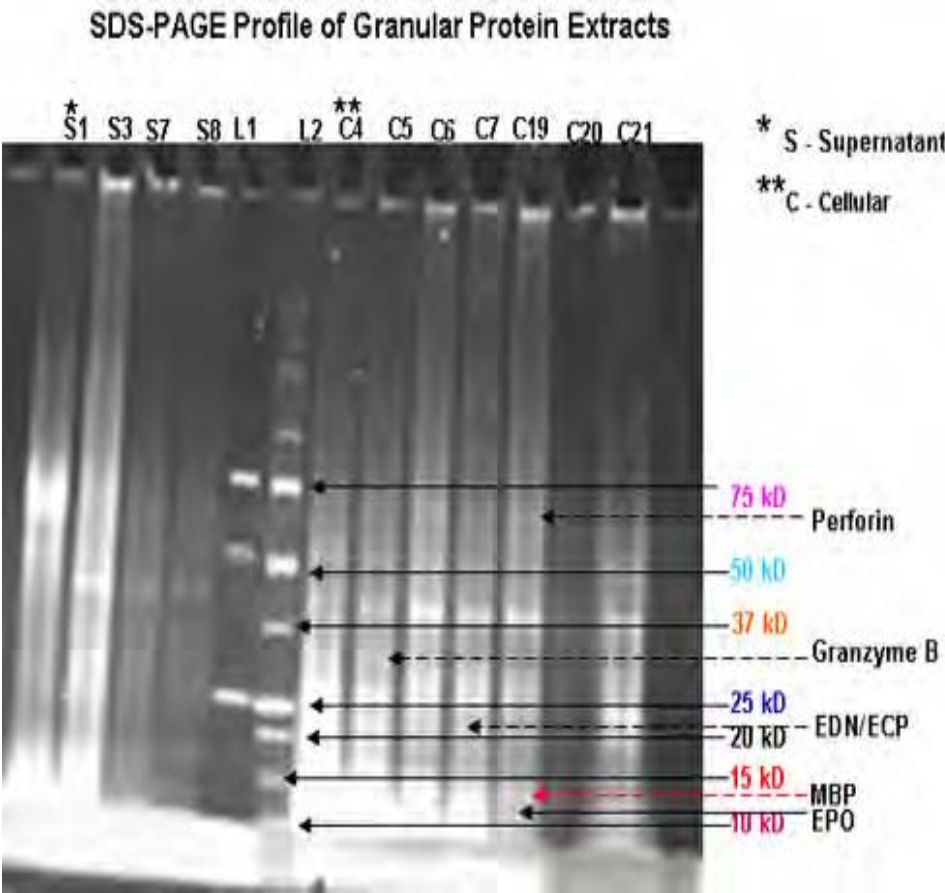
**Figure 1. *Eosinophil cell Lines in Culture.*** Eosinophil cell lines were retrieved from liquid nitrogen, cultured and frozen off to ensure that these cells would be available for use. Photomicrographs of cell line GRC.014.22 were taken (day 1 and day 4). All eosinophil cell lines had the same growth characteristics.

Figure 2.



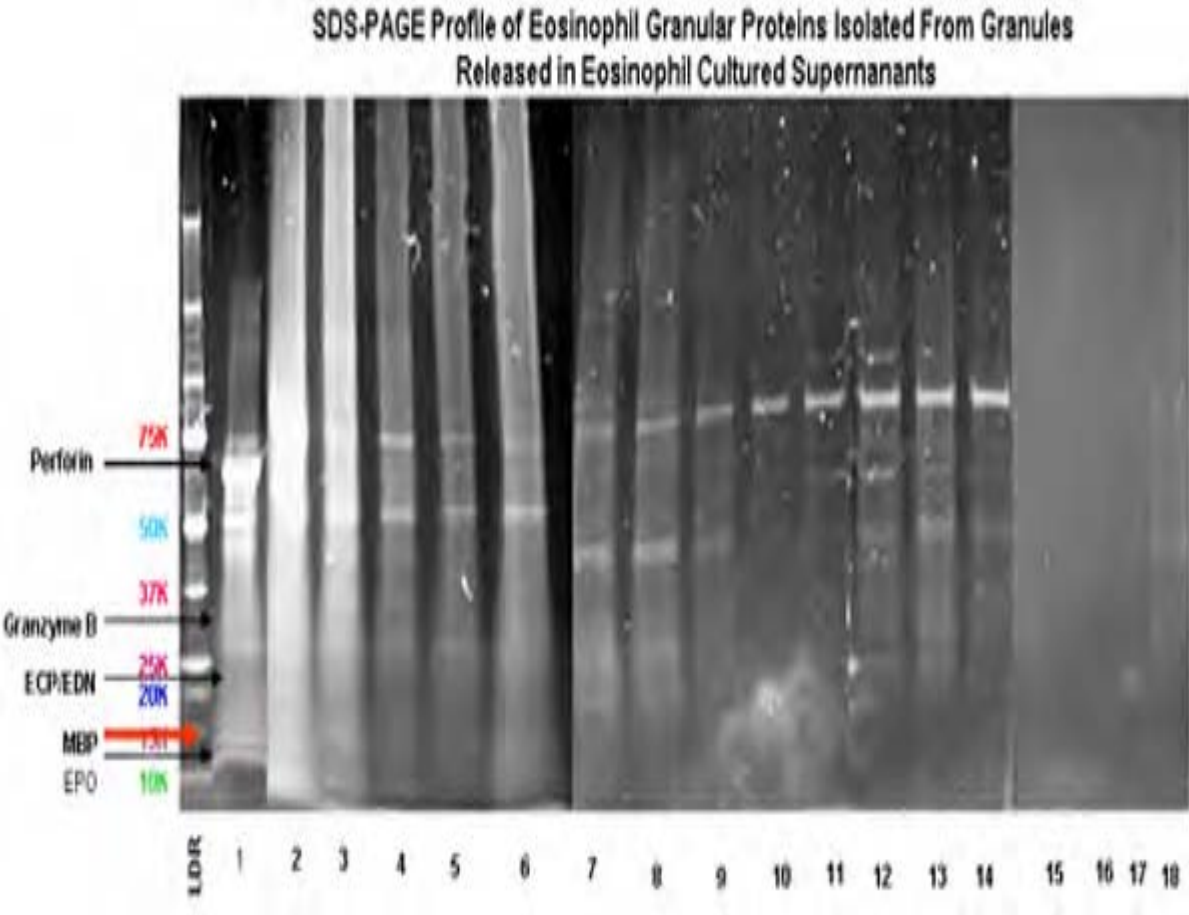
**Figure 2. *Electrophoresis profile of proteins in granules from eosinophil cell line conditioned supernatants.*** Fourteen HCl extracts from granules harvested from supernatants of eosinophil cell line cultures (72 hrs). Based on the molecular size, granular proteins, MBP, EPO, EDN, ECP Granzyme B, and Perforin can be detected. Perforin is seen in all fractions; granzyme b in fractions 1- 6, MBP in early (1- 4) and late (10- 14) fractions; EDN/ECP in fractions 1-3 and 6-8 and EPO in early fractions (1-3).

Figure 3.



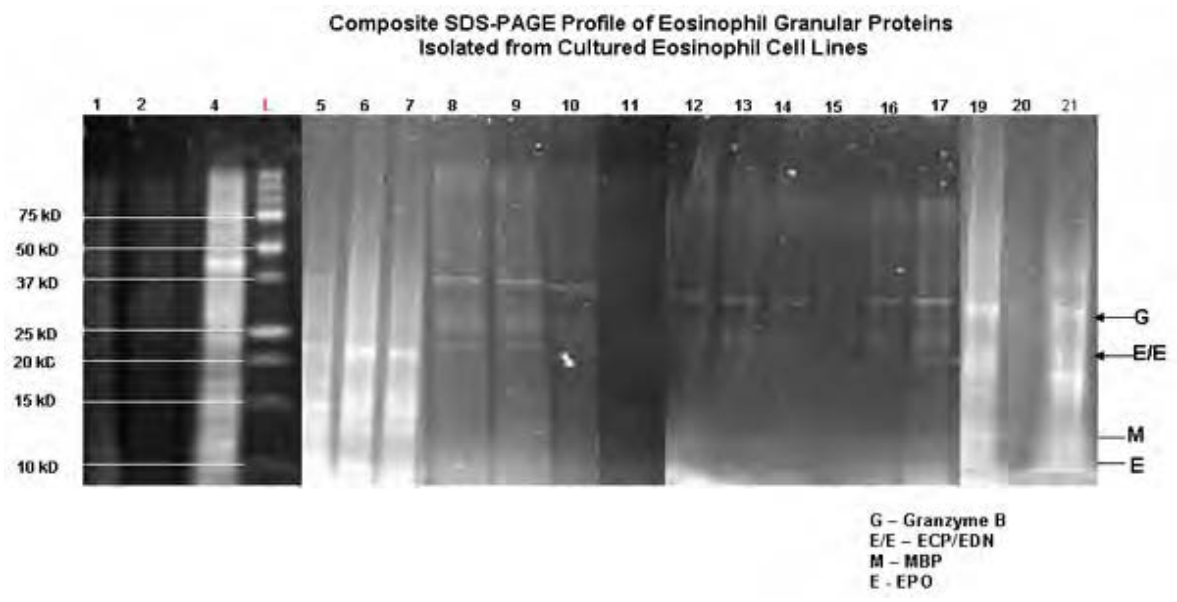
**Figure 3. SDS PAGE profile of proteins in granular isolates from eosinophil cell lines cultured for 72 hrs and 37C.** The molecular weight of the ladder is color coded. With regards to the cellular fractions, perforin was present in fractions 4-7, 19 and 21; granzyme b, perforin, MBP, EDN/ECP and EPO were all found in cellular fractions 4-17, 19 and 21.

Figure 4.



**Figure 4. SDS-PAGE Profile of Eosinophil Granular Proteins Isolated from Granules Released in Eosinophil Cultured Supernatants.** Eighteen HCL-extracted fractions were collected from released granules in eosinophil cultured supernatants. This figure is a composite profile of proteins in each fraction. For fractions 1-14, the results are the same as described in figure 2. There were no bands found in fractions 15-17, however perforin and granzyme b were detected in fraction 18.

Figure 5.

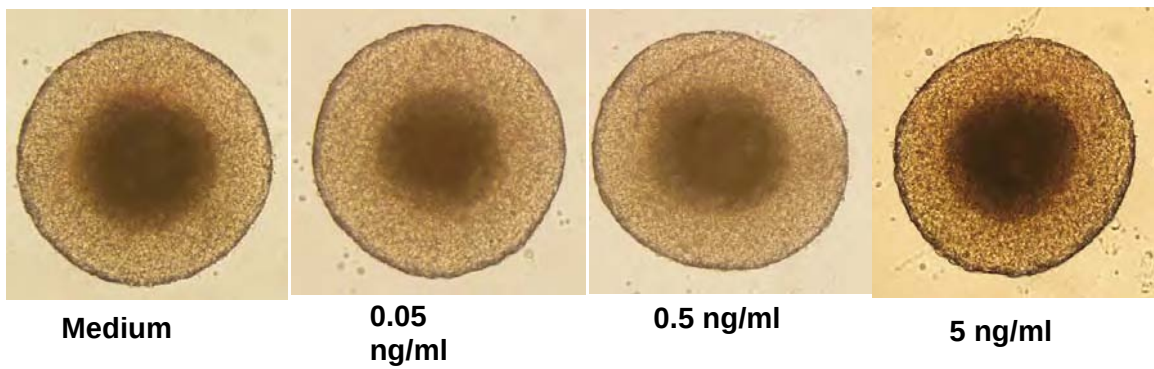


**Figure 5. Composite of SDS-PAGE Profiles of Eosinophil Proteins Isolated from Eosinophil Cell Lines.** Perforin was detected in fractions 1, 2, 4-10, (13, 16, 17, 19) and 21; granzyme b in fractions 1 – 17 and 12; EDN/ECP in fractions 5-10, 12 – 17, 19 and 21; MBP in fractions 4 – 7, 19 and 21; and EPO in fractions 1, 4 -7, 19 and 21.

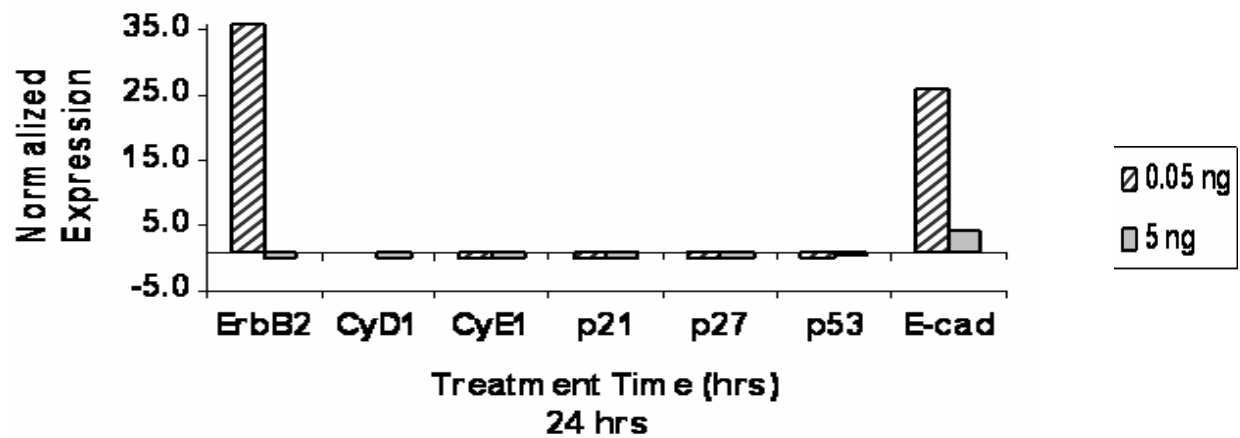
**Figure 6**

**A.**

**EFFECT OF TNF-ALPHA ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(24 Hrs)**



**B.**

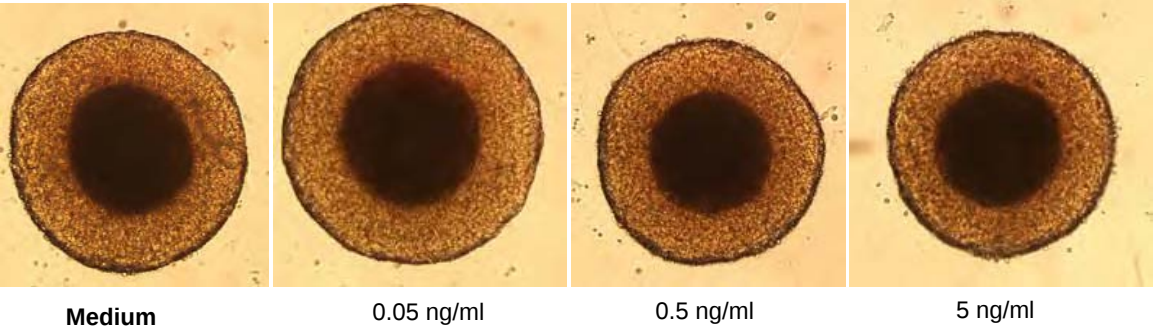


**Figure 6. *Effect on TNF-alpha on Oncogene Expression in MCF-MTS (24 hrs).*** At 24 hrs TNF-alpha treated spheroids began to shed cells, 5ng/ml had the greatest affect. (It was not determined whether these cells were alive or dead) (Figure 6A). At 0.05 ng/ml TNF-alpha dramatically enhanced the expression of erbB2 and e-cadherin but downregulated (1-fold equally) CyD1, CyE1, p21, p27 and p53. At 5 ng/ml all gene expression except e-cadherin, was slightly downregulated. E-cadherin was upregulated 5-fold (Figure 6B).

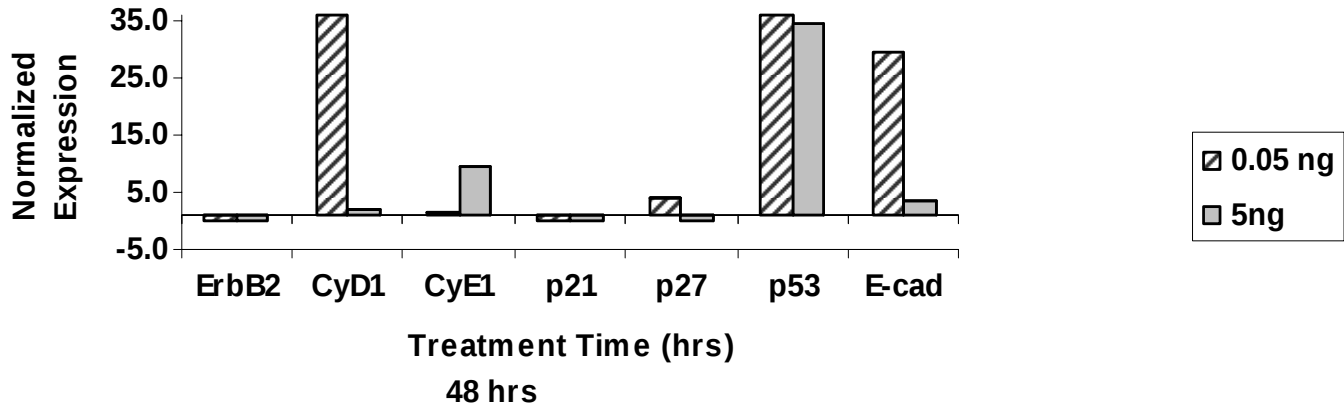
**Figure 7.**

**A.**

**EFFECT OF TNF-ALPHA ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(48 Hrs)**



**B.**

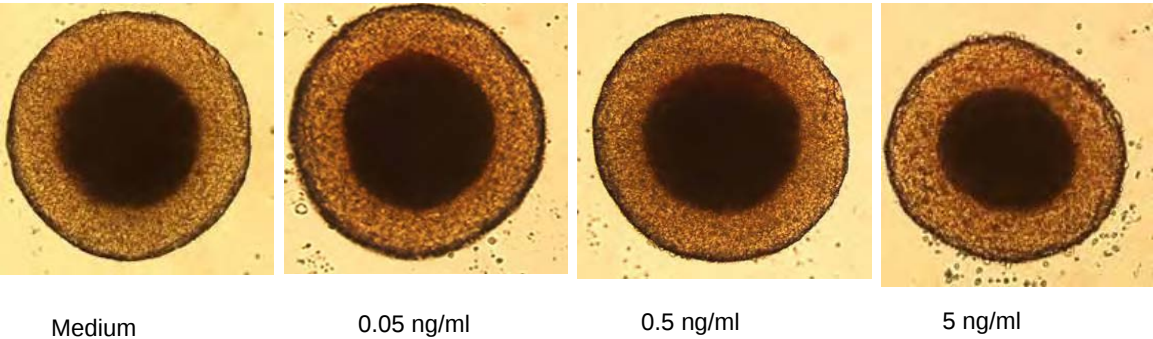


**Figure 7. *Effect of TNF-alpha on Oncogene Expression in MCF-7 MTS (48 hrs).*** At 48 hrs TNF-alpha treatment particularly at 0.5ng/ml and 5ng/ml caused an increased loosening of the cells on the borders of the spheroid resulting in a greater release of cells (Figure 7A). ErbB2 was downregulated by both 0.05 ng and 5 ng, but CyD1 and p53 were dramatically upregulated in expression by 0.05 ng. The effect on e-cadherin was unchanged, upregulated by both concentration > 25-fold by 0.05 ng 5-fold by 5 ng and (Figure 7B).

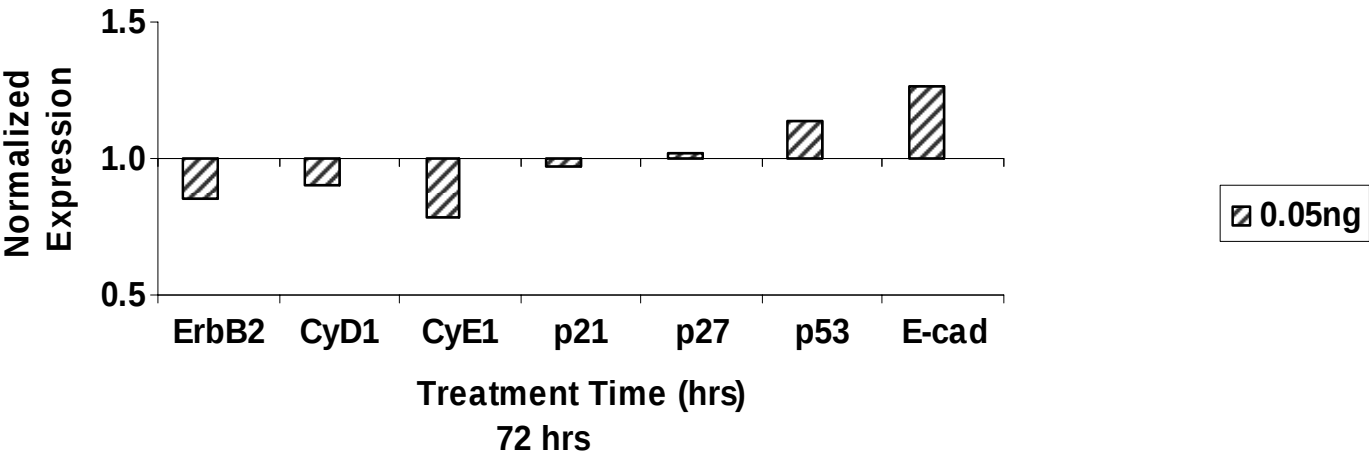
**Figure 8.**

**A.**

**EFFECT OF TNF-ALPHA ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(72 HRS)**



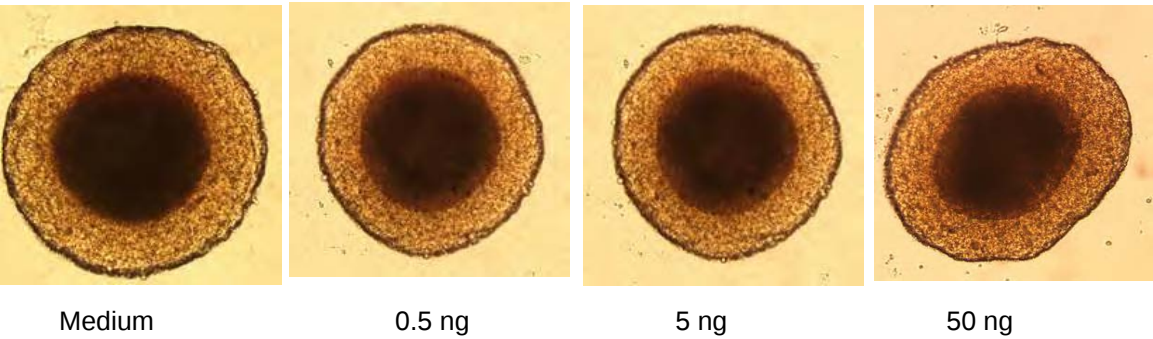
**B.**



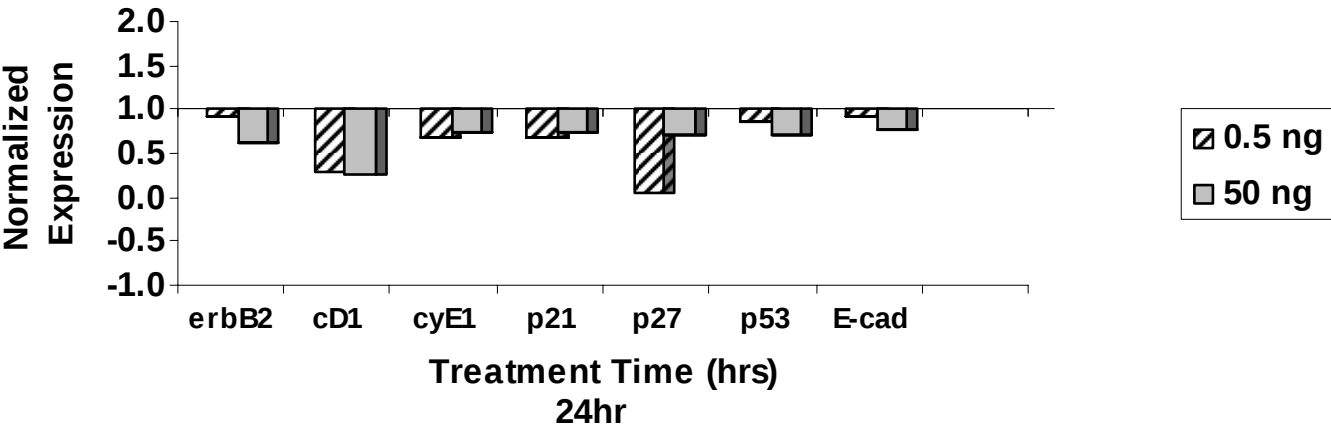
**Figure 8. *Effect of TNF-alpha on Oncogene Expression in MCF-7 MTS (72 hrs).*** At 72 hrs, TNF-alpha causes tremendous destruction of the spheroid border particularly at the highest concentration (5ng/ml) (Figure 8A). At 0.05ng/ml ErbB2, CyD1, CyE1 and p21 were downregulated while p27, p53 and e-cadherin were upregulated. E-cadherin by 1-fold which was > p53 > p27 (Figure 8B). (50 ng was not tested)

**Figure 9.**

**A. EFFECT OF IL-4 ON ONCOGENE EXPRESSION IN MCF-7 MTS (24 HRS)**



**B.**

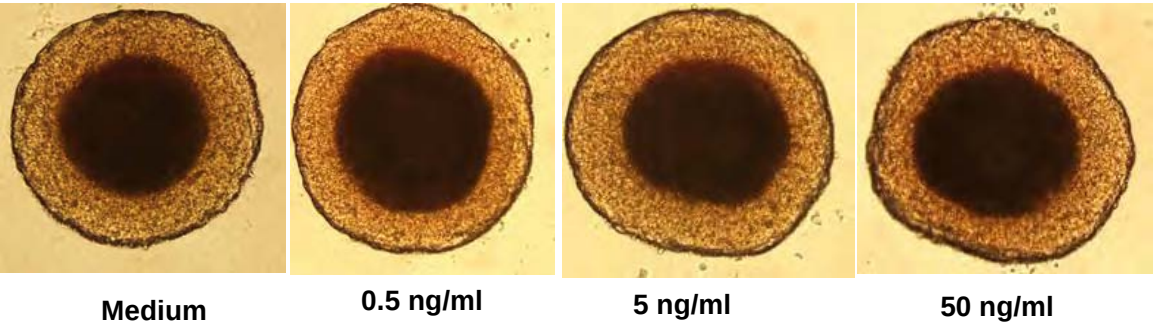


**Figure 9A. *Effect of IL-4 on Oncogene Expression in MCF-7 MTS (24 hrs).*** IL-4 at 24 hrs at 10-fold higher concentrations than TNF-alpha showed minimal disruption of the spheroid surface. 50 ng concentration causes a slight weakening of the spheroid border (Figure 9). At 0.5 ng and 50 ng, IL-4 downregulated all oncogenes tested, CyD1 and p27 1.5-fold greater than erbB2, CyE1, p21, p53 and E-cad (Figure 9B). (N-cadherin not tested)

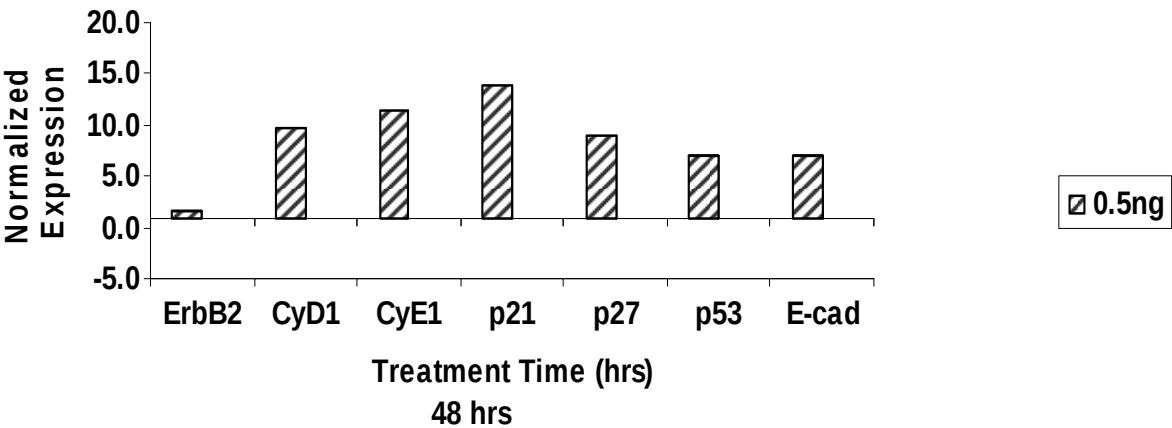
**Figure 10.**

**A.**

**EFFECT OF IL-4 ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(48 HRS)**



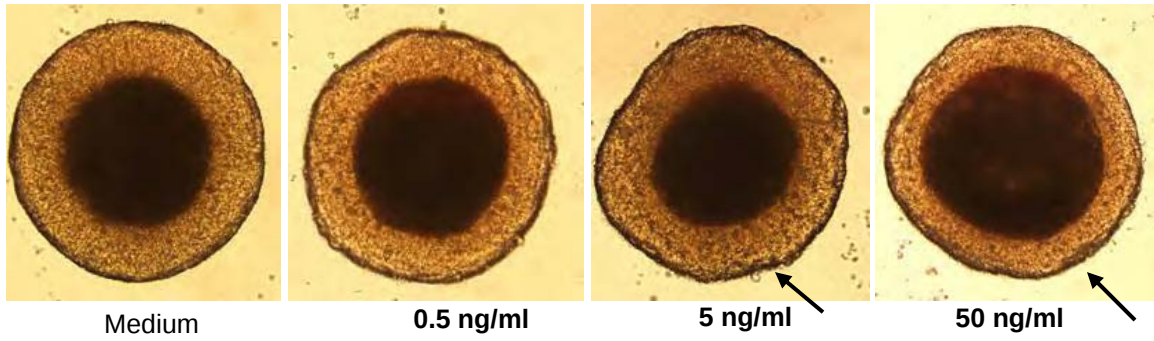
**B.**



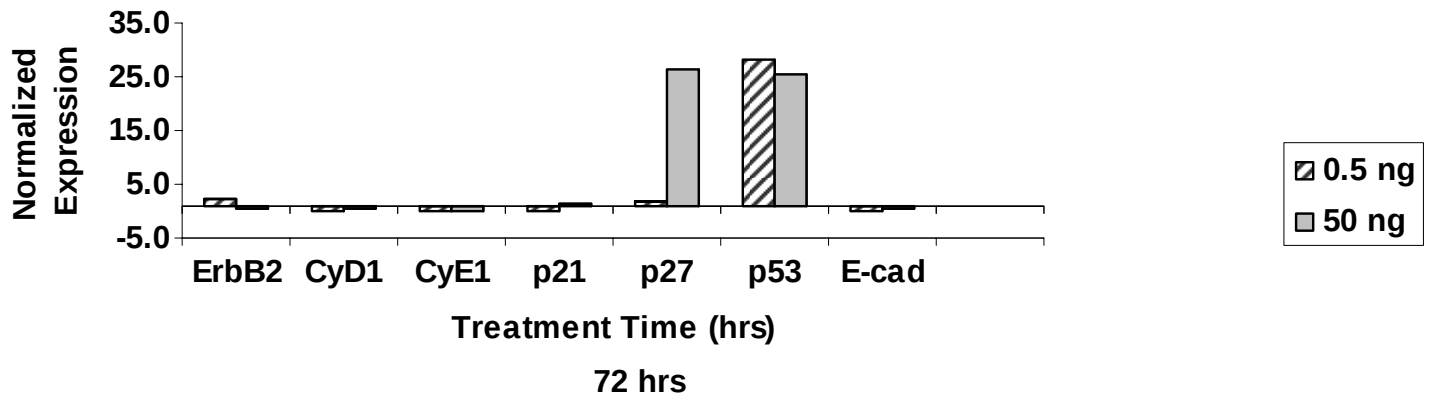
**Figures 10A.** *Effect of IL-4 on Oncogene Expression in MCF-7 MTS (48 hrs).* At 48 hrs the effect on MTS growth did not change (Figure 10A). At 48 hrs IL-4 at 0.5 ng/ml dramatically upregulated the expression of all genes tested, ErbB2 1-fold (Figure 10B).

**Figure 11.**

**A. EFFECT OF IL-4 ON ONCOGENE EXPRESSION IN MCF-7 MTS (72 hrs)**



**B.**

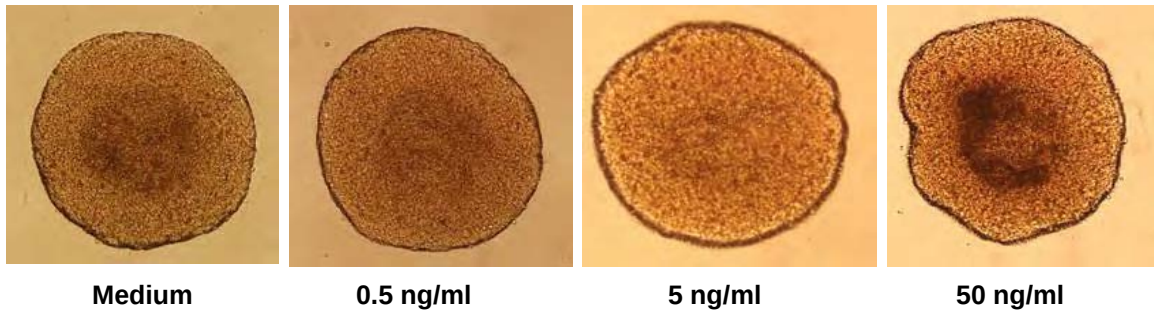


**Figure 11A. Effect of IL-4 on Oncogene Expression in MCF-7 MTS (24 hrs).** At 72 hrs, IL-4 begins to destroy the integrity of the spheroid ( 5 ng and 50 ng) (Fig. 11A). Oncogenes p27 at 50 ng and p53 at 0.5 and 50 ng shows enhanced expression (25-fold), while CyD1, CyE1 and e-cadherin were downregulated 1-fold by both concentrations. ErbB2 was up regulated by 0.5 ng but downregulated by 50 ng. With p21 the opposite effect was observed, i.e., low concentration 0.5 ng downregulated expression while high concentration, 50 ng, upregulated expression equally 1-fold (Figure 11B).

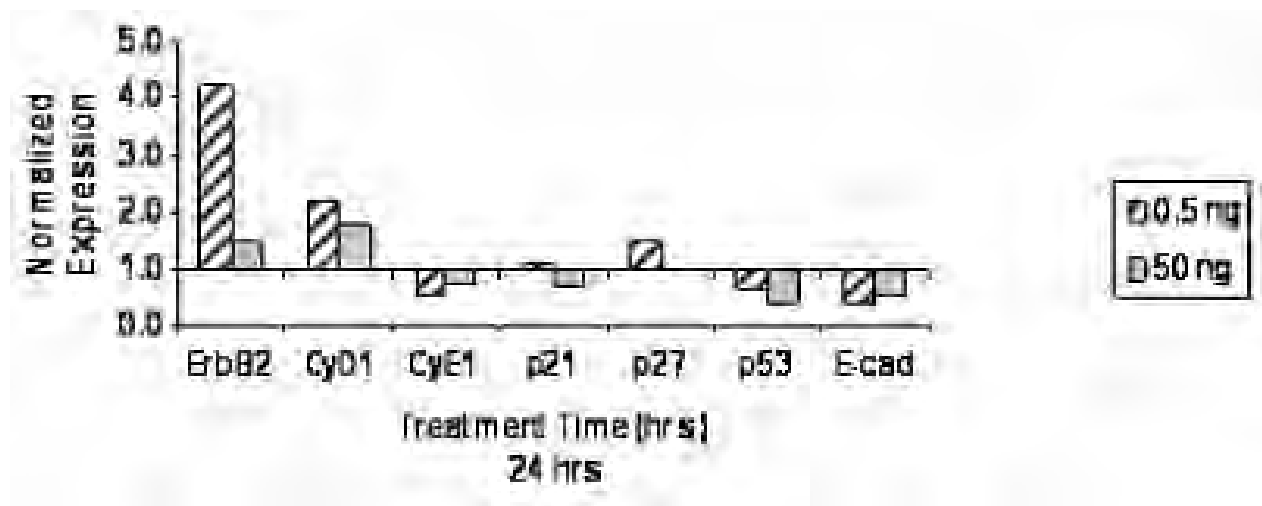
**Figure 12.**

**A.**

**EFFECT OF IL-10 ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(24 HRS)**



**B.**

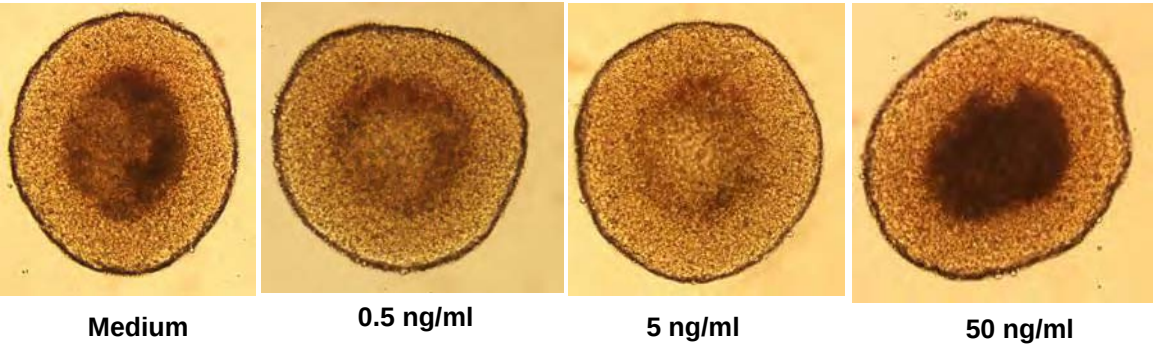


**Figure 12A. *Effect of IL-10 on Oncogene Expression in MCF-7 MTS (24 hrs).*** IL-10 had no apparent affect on MCF-7 spheroid growth (Figure 12A). IL-10 had a variable effect on oncogene expression. It upregulated both erbB2 and CyD1 with both low and high concentration, ErbB2 4-fold (0.5 ng) and CyD1 2-fold (0.5 ng). CyE1, p53 and e-cadherin were downregulated by both low and high concentrations (Figure 12B).

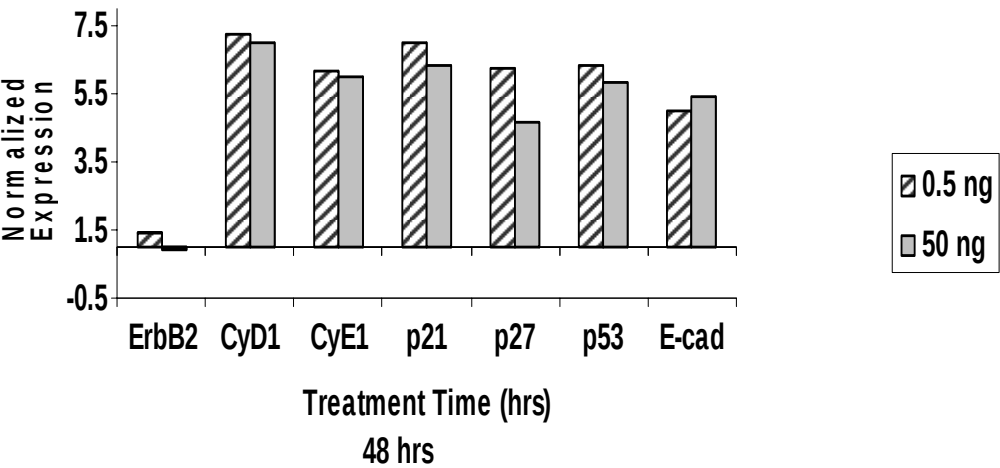
**Figure 13.**

**A.**

**EFFECT OF IL-10 ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(48 HRS)**



**B.**

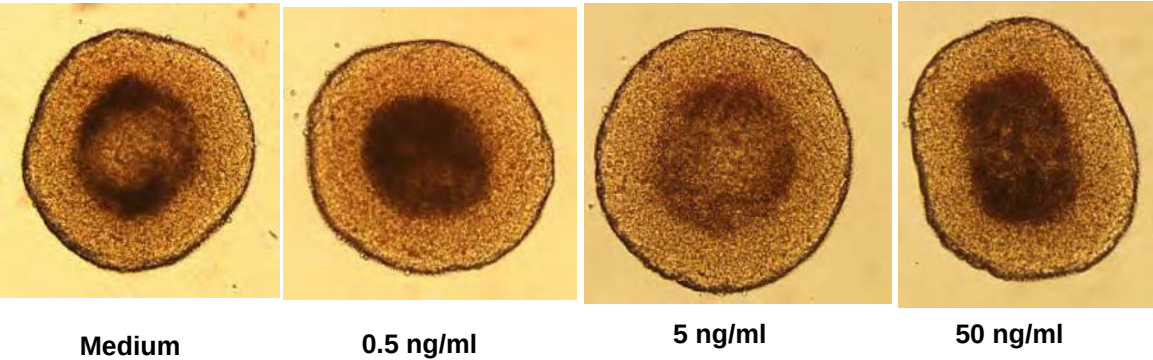


**Figures 13A.** *Effect of IL-10 on Oncogene Expression in MCF-7 MTS (48 hrs).* High dose treatment (50 ng/ml) induced a high level of necrosis and the spheroid border is being compromised (Figure 13A). Both low and high concentrations enhanced the expression of all genes by 5-7 fold except erbB2 which was upregulated by 0.5 ng (1-fold) but slightly downregulated by 50 ng (Figure 13B).

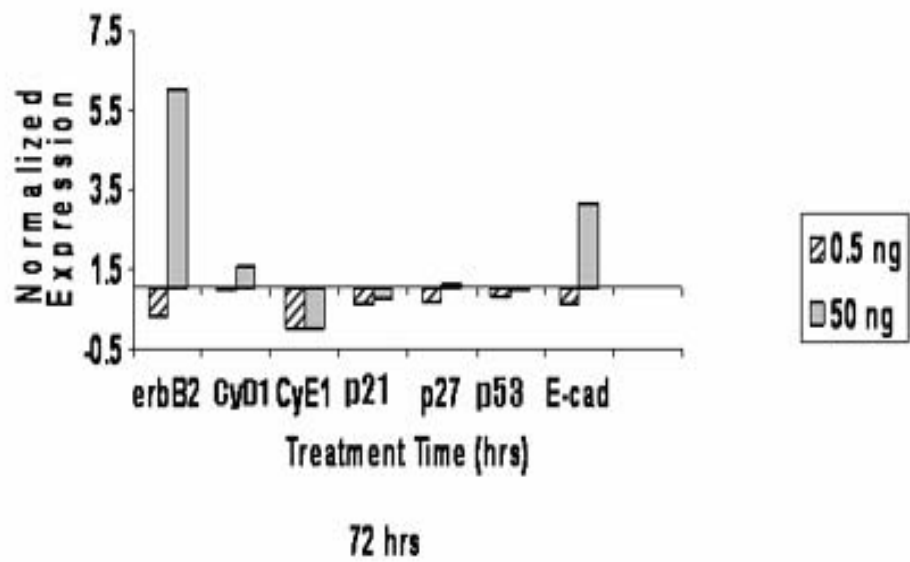
Figure 14.

A.

EFFECT OF IL-10 ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(72 HRS)



B.

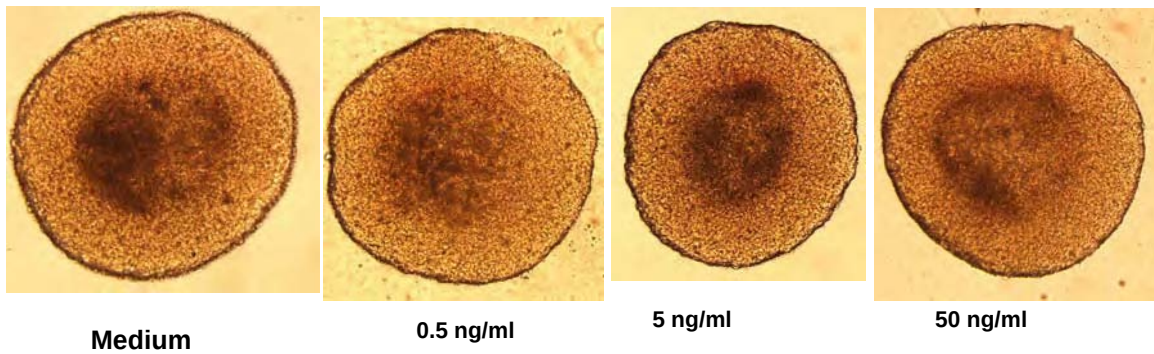


**Figure 14A.** *Effect of IL-10 on Oncogene Expression in MCF-7 MTS (72 hrs).* At 72 hrs necrosis appears in both control and test spheroids (Figure 14A). For genes, erbB2, CyD1, p27 and e-cadherin, low dose IL-10 (0.5 ng) downregulated expression, while high dose (50 ng) upregulated expression. With CyE1, p21 and p53 both concentrations downregulated expression (Figure 14B).

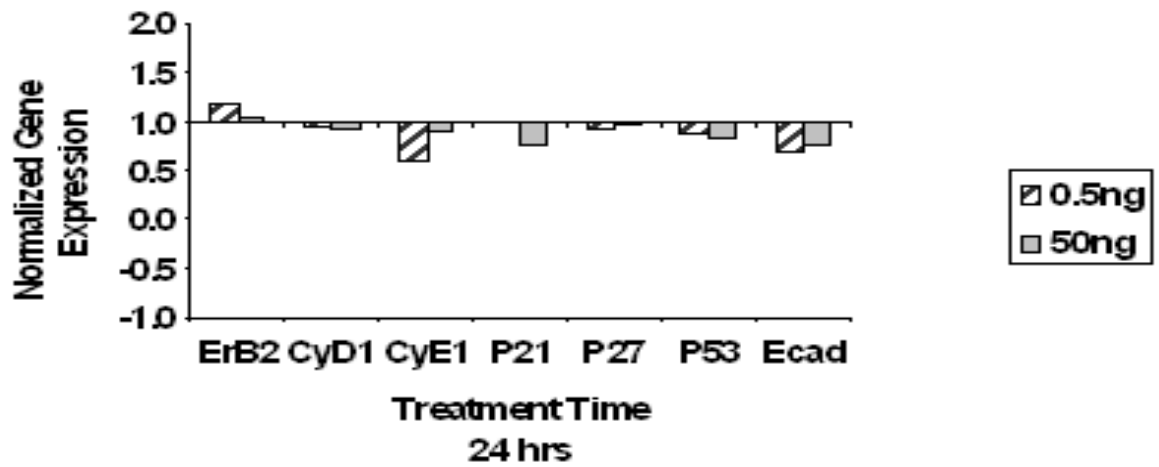
**Figure 15.**

**A.**

**EFFECT OF IL-12 ON ONCOGENE EXPRESSION IN MCF-7MTS  
(24 HRS)**



**B.**

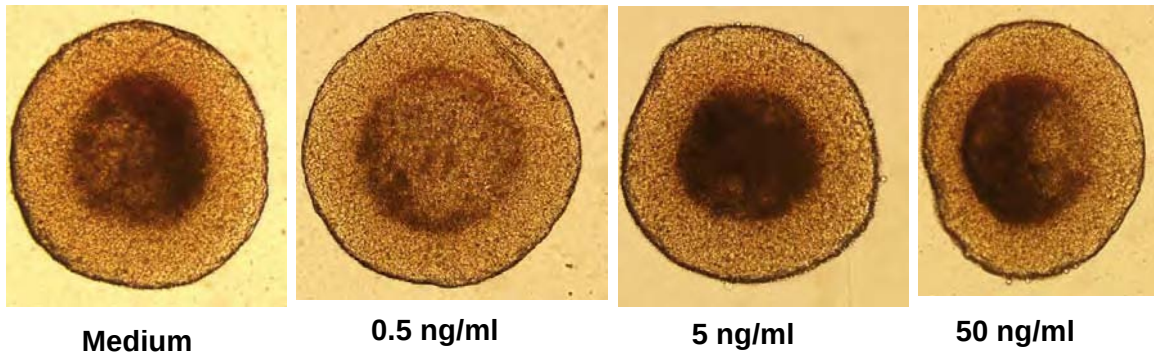


**Figure 15 A. *Effect on IL-12 on Oncogene Expression in MCF-7 MTS (24 hrs).*** IL-12, like IL-10 had little to no effect on the integrity of the spheroids and no demonstrable necrotic effects over that of the medium-treated spheroids (Figure 15A). With regards to gene expression, all genes except erbB2 were downregulated by both low and high concentrations (Figure 15B).

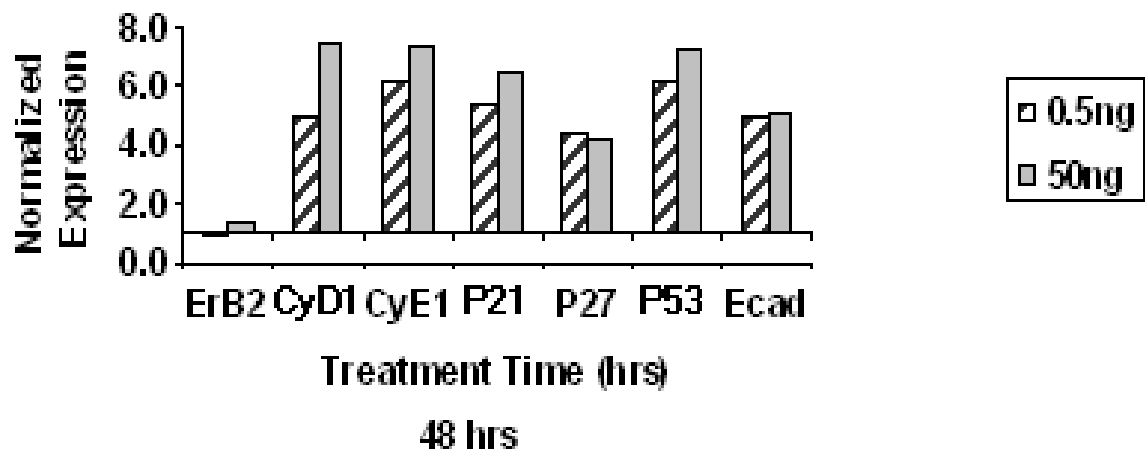
**Figure 16.**

**A.**

**EFFECTS OF IL-12 ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(48 HRS)**



**B.**

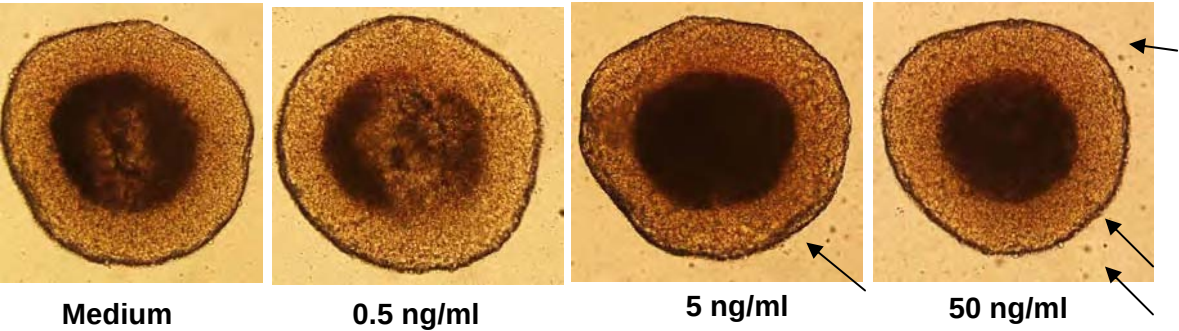


**Figures 16A. *Effect of IL-12 on Oncogene Expression in MCF-7 MTS (48 hrs).*** At 48 hrs necrosis appears in treated and untreated spheroids and there is some evidence of destruction at the borders, particularly with 5 ng and 50 ng treatment (Figure 16A). IL-12, like IL-10 (48 hrs) dramatically enhanced (> 4-fold) the expression of all genes except erbB2 which was minimally enhanced by 50 ng (Figure 16B).

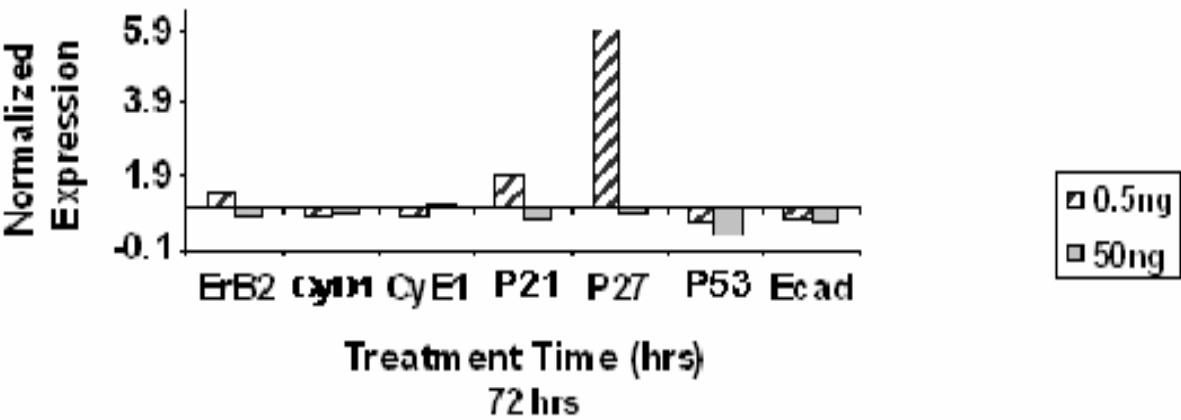
Figure 17.

A.

EFFECT OF IL-12 ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(72 HRS)



B.

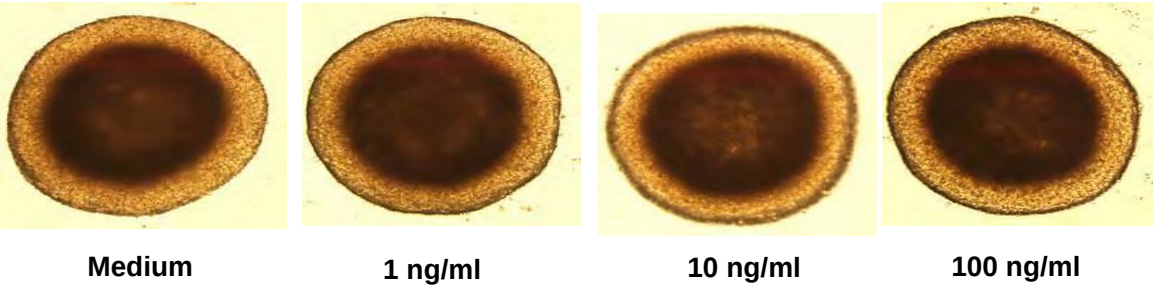


**Figures 17A.** *Effect of IL-12 on Oncogene Expression in MCF-7 MTS (72 hrs).* At 72 hrs there is increasing necrosis in both treated and untreated spheroids and greater evidence of destruction the spheroid borders (arrows) (Figure 17A). ErbB2, p21 and p27 were upregulated by low dose IL-12 (0.5 ng), p27 by 5.9- fold, p21 by 1.9- fold and erbB2 < 1- fold. All other genes were downregulated by both low and high dose treatments (Figure 17B).

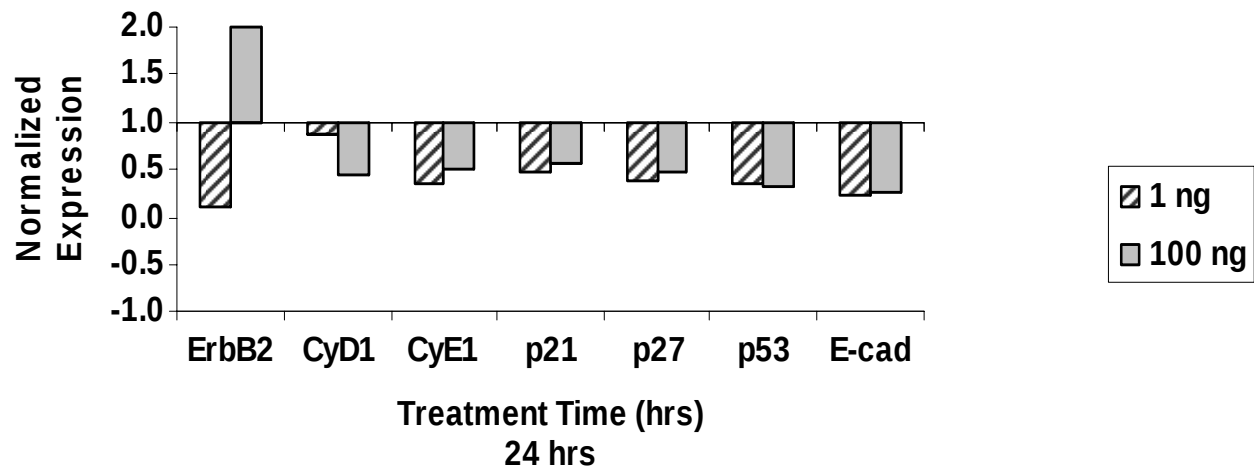
**Figure 18.**

**A.**

**EFFECT OF MBP ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(24 HRS)**



**B.**

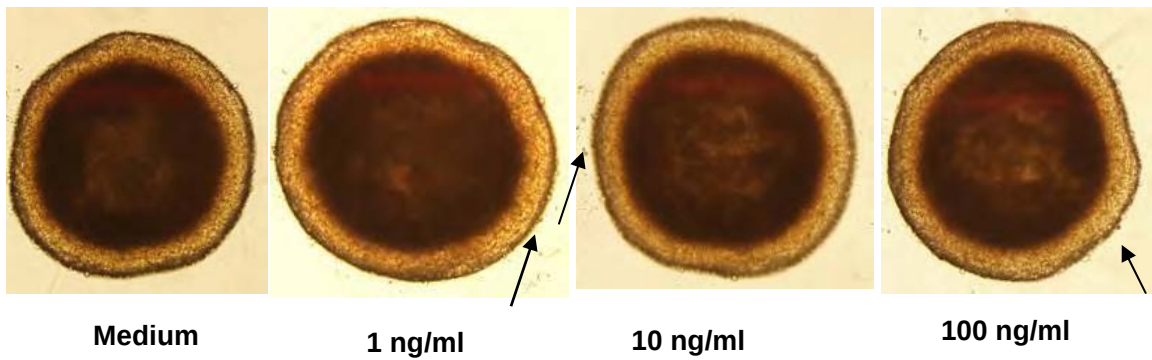


**Figures 18A. *Effect of MBP on Oncogene Expression in MCF-7 MTS (24 hrs).*** At 24 hrs, an immediate effect on the spheroid borders. Detached cells can be observed particularly with 100 ng (Figure 18A). MBP downregulated all genes at both concentrations except erbB2 which was enhanced 2-fold by the high concentration (100 ng) (Figure 18B).

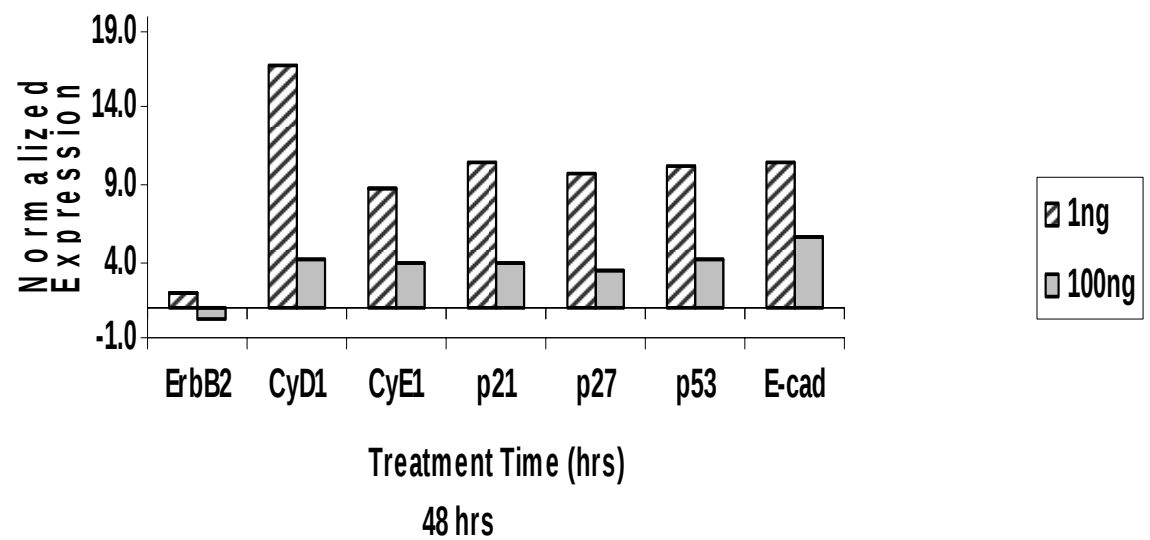
**Figure 19.**

**A.**

**EFFECT OF MBP ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(48 HRS)**



**B.**

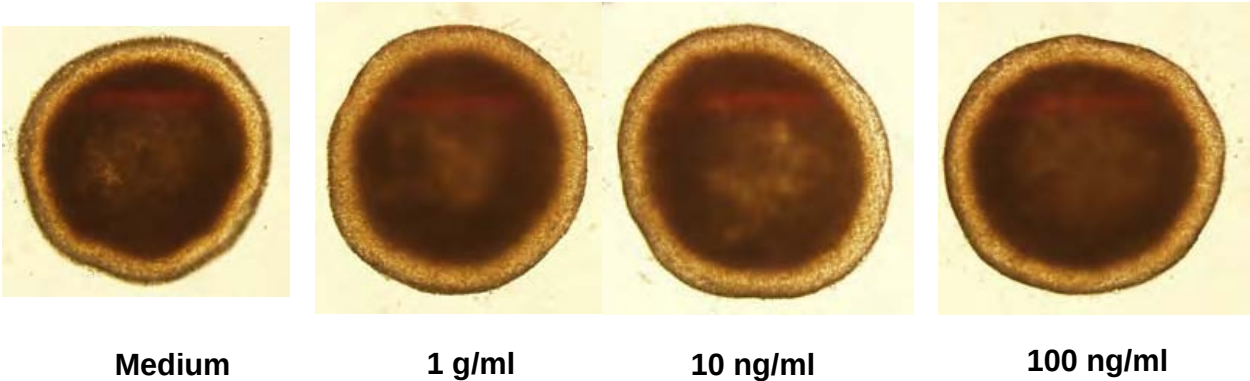


**Figure 19A.** *Effect of MBP on Oncogene Expression in MCF-7 MTS (48 hrs).* Detachment of cells continues at 48 hrs (arrows) (Figure 19A). All genes are upregulated at high levels ( $> 4$  - fold), except erbB2 which is enhanced (2- fold) by 1 ng and downregulated by 100 ng (1- fold) (Figure 19B).

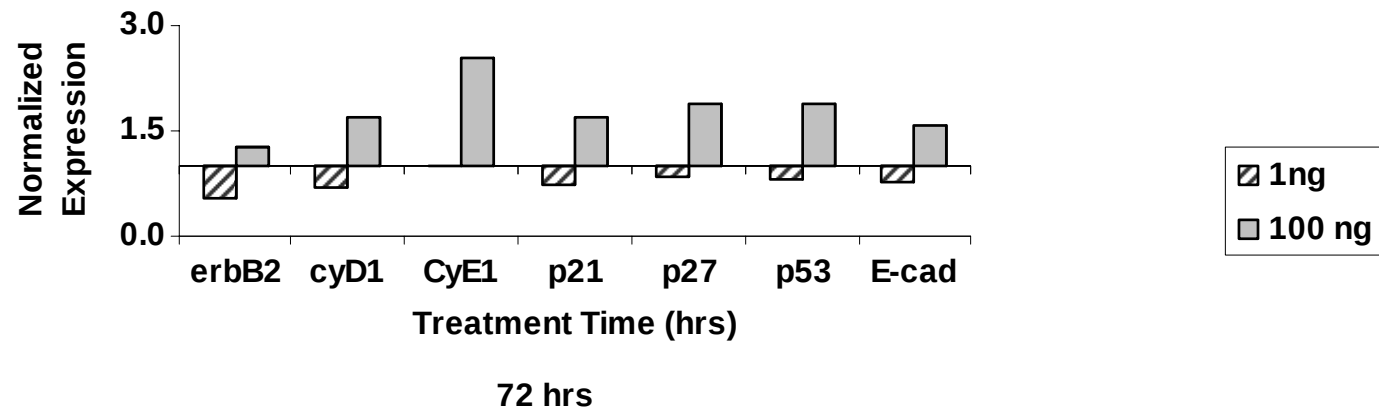
**Figure 20.**

**A.**

**EFFECT OF MBP ON ONCOGENE EXPRESSION IN MCF-7 MTS  
(72 HRS)**



**B.**



**Figures 20 A. *Effect of MBP on Oncogene Expression in MCF-7 MTS (72 hrs).***  
There were no additional distinguishing affects on spheroid growth observed at 72 hrs (Figure 20A). At 72 hrs 100 ng enhanced oncogene expression ranging from 1-fold (erbB2), 2.5-fold (CyEl) and 1 ng down regulates all genes, except CyEl which showed no effect (Figure 20B).