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PRINCIPAL INVESTIGATOR: Susan A. Chrysogelos, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

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13. ABSTRACT (Maximum 200) Apoptosis is a normal cellular process involving a specific series of events leading to the death of a cell. In the nude mouse, MCF-7 human breast cancer cells deprived of estrogen undergo this process, but MCF-7 cells grown <i>in vitro</i> respond to estrogen withdrawal by accumulating in G ₁ rather than dying. This suggests that stromal-epithelial interactions are critically involved in the signalling process that induces apoptosis. The goal of this project is to develop model systems using coculture of breast epithelial cells and fibroblasts that will mimic the <i>in vivo</i> induction of programmed cell death. Our work thus far indicates that both stromal-epithelial signaling and the influence of basement membrane components are important for antiestrogens to induce apoptosis rather than cell cycle arrest, and that there is a secreted signal from normal fibroblasts that can induce apoptosis in MCF-7 cells. This model system will not only provide a useful alternative to animal models for evaluating the cytotoxic (as opposed to cytostatic) effects of potential therapeutic agents, but will also be a valuable tool for us in elucidating the signals that are responsible for programmed cell death in breast cancer cells with a future goal of manipulating this process to provide new treatments.				
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James A. Chappell 11/22/96
PI - Signature Date

**THE ROLE OF CELL-CELL SIGNALING IN THE INDUCTION
OF APOPTOSIS IN BREAST CANCER CELLS**

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THE ROLE OF CELL-CELL SIGNALING IN THE INDUCTION OF APOPTOSIS IN BREAST CANCER CELLS

INTRODUCTION

Apoptosis, or programmed cell death, is a normal and important mechanism by which cell populations are regulated in multicellular organisms. This process occurs in many cell and tissue types, including neurons, erythroid and lymphoid cells, the thymus, prostate, and uterus. In the mammary gland, apoptosis occurs during involution when there is massive tissue remodeling following the cessation of lactation. In programmed cell death (as opposed to necrotic death) there is a rapid, sustained increase in intracellular Ca^{2+} that leads to chromatin fragmentation by a Ca^{2+} dependent nuclease into units that are multimers of nucleosomes (thus producing a DNA ladder that is the hallmark of apoptosis) (1). The resulting nuclear collapse produces a cell with a convoluted, or "blebbed", surface as the nucleus and the cell break into several membrane-bound fragments, or apoptotic bodies, that are subsequently phagocytosed (2). Apoptosis has been observed in numerous organisms and cell types, and in response to a variety of stimuli (3). While the morphological characteristics of apoptosis are reasonably well defined, the molecular mechanisms underlying them are more elusive. In most cells, RNA and protein synthesis are required for, and inhibitors of these processes can prevent, apoptosis (3). Additionally, several genes have been implicated as having a role in regulating the apoptotic process including p53, myc, fos, bcl-2 and TRPM-2 (SGP-2 or clusterin) (4-7).

In some cell types such as immature T cells, the process of apoptosis can occur *in vitro*. However other cases, such as estrogen withdrawal in breast cancer cells, result in programmed cell death only *in vivo*. When MCF-7 tumors in nude mice are subjected to estrogen ablation the tumors regress, accompanied by the rapid induction of TRPM-2 and the appearance of the DNA ladder that is characteristic of apoptosis (8). However withdrawal of estrogen from MCF-7 cells cultured *in vitro* results in the accumulation of cells in G_1 , with no net reduction in cell number (9). Thus the same treatment is cytotoxic *in vivo* but only cytostatic *in vitro*. Clearly, there is a signaling process in the solid tumor *in vivo* that is lacking *in vitro*. Observations such as these in both breast and prostate have led to the proposal that apoptosis may be mediated by stromal-epithelial interactions and/or basement membrane components in these cell types.

Purpose of the Present Work

*The goal of cancer therapies is to produce cytotoxic effects (killing of tumor cells) rather than cytostatic effects (inhibition of growth). Studies of apoptosis suggest that specific stromal-epithelial interactions and/or basement membrane components likely provide signals that result in the death of breast cancer cells. The objectives of this project are to develop an *in vitro* culture system that mimics the interactions leading to programmed cell death *in vivo* upon estrogen ablation, and to use this model system to investigate the intercellular signals responsible for mediating programmed cell death in breast cancer cells with the hope that in the future new strategies can be developed to induce programmed cell death and exploit this normal cellular mechanism for limiting growth. Additionally, the development of this *in vitro* model system will also provide a valuable alternative to animal models for testing the cytotoxic effects of potential therapeutic agents.*

Methods of Approach (as stated in the original proposal)

1. **Determine the requirements for apoptosis to occur in vitro following estrogen withdrawal.** A model system is being developed using co-culture of normal or tumor breast fibroblasts with MCF-7 breast cancer cells on plastic alone or on Matrigel (basement membrane) or Vitrogen. Apoptosis will be assessed by the appearance of DNA ladders, TRPM-2 expression, TUNEL assays, a cell death detection ELISA kit (Boehringer Mannheim), and microscopy to identify individual apoptotic cells.
2. **Use the in vitro model system to elucidate the signals that are responsible for the induction of programmed cell death in breast cancer cells.** By culturing breast epithelial and stromal cells using Transwell dishes it will be determined if cell-cell contact is required for apoptosis to occur or if there is a secreted signal. Conditioned media will also be used to assess the role of a secreted factor.

RESULTS

As this is the final report for this grant, we have included our results for both year 1 (as previously reported) and year 2.

Development of experimental model systems for studying the induction of programmed cell death in vitro (Task 1; see Appendix for copy of original Statement of Work)

To design an *in vitro* model for programmed cell death in breast cancer, we have focused on providing an environment that restores the signals normally present *in vivo* that are needed for this process to occur. In preliminary experiments, we cocultured MCF-7 breast cancer cells with normal breast fibroblasts, and then subjected the cells to estrogen withdrawal for several days. Analysis of the DNA for nucleosomal ladders revealed only a continuous smear of degraded DNA from cells at 8 hours post estrogen withdrawal. However, the extent of DNA degradation was considerably greater in MCF-7 cells cocultured with fibroblasts than in MCF-7 cells cultured alone and subjected to the same treatment. Additionally, at 5 days post-estrogen withdrawal, virtually no intact DNA or RNA was recovered from the cocultured cells, while MCF-7 cells cultured alone showed no further degradation. These results suggest that by coculturing breast epithelial and stromal cells we have taken the first step in rendering the cells susceptible to the toxic effects of estrogen withdrawal, but these conditions do not allow for induction of the apoptotic phenotype that includes nucleosomal laddering. This is consistent with recent reports that treatment of breast cancer cells *in vitro* with the antiestrogens tamoxifen or toremifene may be able to induce some of the features of apoptosis, but not nucleosomal fragmentation of DNA (10). Additionally, there is accumulating evidence that apoptosis is not uniform in phenotype: there may be different pathways that result in different phenotypic manifestations, such as nucleosomal laddering vs. a less specific fragmentation of DNA.

We further pursued this line of investigation by coculturing MCF-7 cells and breast fibroblasts, treating with 10^{-7} M ICI 164,384 (a pure anti-estrogen) to block estrogen action, and performing TUNEL assays (11) in order to identify individual apoptotic cells. This assay involves the labeling of nicks in the DNA within the cells using terminal deoxy-transferase and biotinylated dUTP. By coculturing the cells in chamber slides and directly staining apoptotic cells within the

chamber slides, we expected that the TUNEL assay would allow *in situ* visualization of cells undergoing apoptosis and would permit us to determine if apoptosis occurs preferentially at points of stromal-epithelial contact. However, we found there was a great deal of cell loss from the slides during the processing of the TUNEL assay (this procedure is generally used on fixed tissue sections). While one might predict that apoptotic cells would be preferentially lost from the slide, there could be other causes as well.

Therefore, we chose to test a cell death detection ELISA manufactured by Boehringer Mannheim. This is a quantitative, photometric "sandwich" enzyme immunoassay for the detection of histone-associated DNA fragments that have been released into the cytoplasm as a result of the induction of apoptosis. As a positive control for the assay we used CEM cells plus and minus dexamethasone treatment (a well-characterized *in vitro* system for the induction of apoptosis). We also chose to test a calcium ionophore (Calcium Ionophore A23187; Sigma) in comparison to ICI treatment for the ability to induce apoptosis in MCF-7 breast cancer cells when cocultured with breast fibroblasts. Such ionophores have been found to bypass the early molecular events in apoptosis and activate the endonuclease responsible for nucleosomal fragmentation. Additionally, such treatments have been effective in inducing apoptosis in prostate cancer cells. To visually confirm apoptosis in individual cells, we turned to fluorescence microscopy using Hoechst 33258 which stains chromatin.

We found that short (4.5 hours) treatment with the calcium ionophore consistently resulted in a modest induction of apoptosis in MCF-7 breast cancer cells, while ICI treatment (4.5 hours or 24 hours) showed only slight effects as measured by the cell death detection ELISA. Membrane blebbing (which is a later marker of apoptosis) could be observed visually at 24 hours calcium ionophore treatment. When MCF-7 cells were cocultured with breast fibroblasts and treated with the calcium ionophore or ICI, there was no significant quantitative difference by ELISA in the induction of apoptosis as compared to MCF-7 cells cultured alone. However, there was a marked difference in the structural organization of the cells. When MCF-7 cells are cultured alone and treated with calcium ionophore, occasional apoptotic cells are found randomly in the field by microscopy. Coculture of MCF-7 cells and breast fibroblasts results in characteristic islands of epithelial cells surrounded by fibroblasts, and with calcium ionophore treatment individual epithelial islands are seen to be either unaffected or entirely apoptotic as evidenced by nuclear condensation and membrane blebbing. These results imply that the structural organization of the cells relative to each other influences the induction of apoptosis.

Studies with normal breast epithelial cells have shown that culture on basement membrane or stromally derived matrices can allow these cells to exhibit a differentiated phenotype not seen when the cells are cultured on plastic. On Matrigel (a murine tumor extract that is rich in basement membrane components), the cells are capable of secreting milk proteins in the presence of lactogenic hormones and of forming spherical structures resembling the alveoli of a lactating mammary gland (12). Coculture of normal mammary epithelial and fibroblastic cell clones in the presence of lactogenic hormones, but without exogenous extracellular matrix components, also was found to result in the synthesis of β -casein (13). However the structures formed by these cells were quite different when grown in type I collagen gels as compared to on plastic alone: on plastic the cells grew in a layer with islands of epithelial cells surrounded by fibroblasts, while

in type I collagen, three-dimensional duct-like structures were formed. *In vivo*, the mammary gland undergoes the process of involution after lactation is complete and lactogenic hormones decrease. During this process, the alveoli collapse, there is destruction of the basement membrane, and epithelial islands become interspersed with stromal cells (14,15). It has also been found that the mechanism by which involution occurs involves apoptosis, complete with DNA fragmentation and induction of TRPM-2, c-myc, and p53 (15). Taken together, these findings suggest that both basement membrane and stromal components are necessary for mediating the signals required for programmed cell death.

Therefore, to determine the effects of these growth conditions on the induction of apoptosis by calcium ionophore or ICI *in vitro*, we compared coculture of MCF-7 breast cancer cells and breast fibroblasts on plastic alone or on Matrigel. Figure 1 shows the results of a cell death detection ELISA performed using the following cell culture conditions and treatments: MCF-7 cells cultured alone on either plastic or Matrigel, breast fibroblasts cultured alone on either plastic or Matrigel, and MCF-7 cells and breast fibroblasts cocultured on either plastic or Matrigel; treatments are control (untreated), calcium ionophore for 24 hours, or ICI for 24 hours. The effects on the level of apoptosis are slight or nonexistent for all treatments and culture conditions with the exception of the MCF-7 cells cocultured with breast fibroblasts on Matrigel. Under these conditions, we not only see a strong induction of apoptosis with calcium ionophore treatment, but also with ICI treatment. These results indicate that both stromal-epithelial signaling and the structural organization that results from the presence of basement membrane components are necessary for induction of apoptosis upon estrogen deprivation.

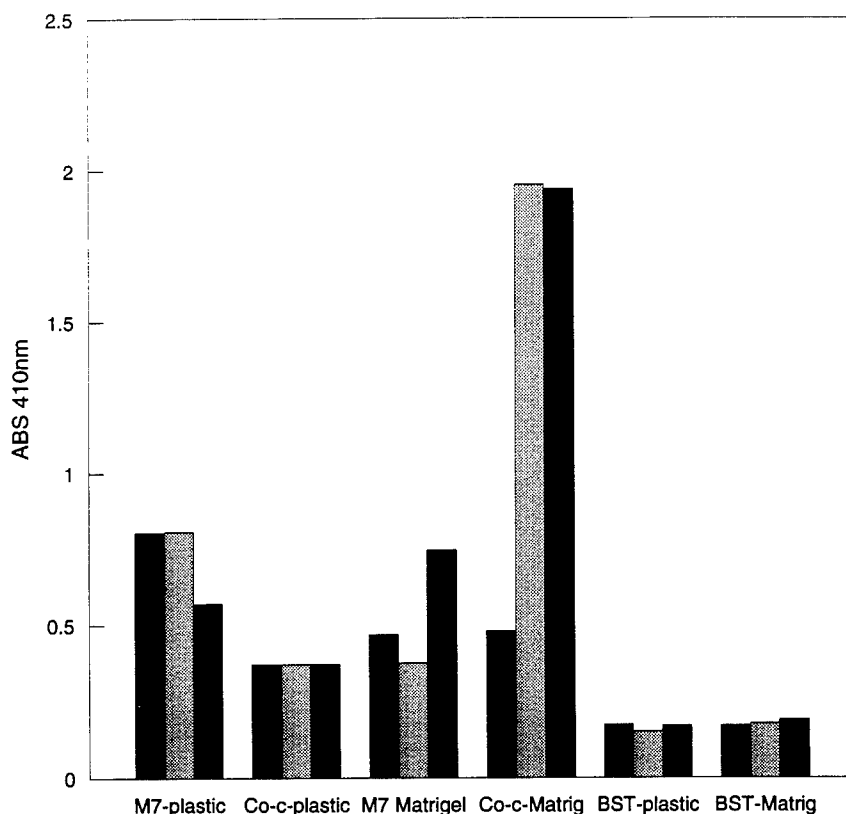


Figure 1. Apoptosis in MCF-7 cells as detected with a Cell Death ELISA. Each group of three consists of control, untreated cells (left), ICI treatment for 24 hrs (middle), and Ca²⁺ ionophore treatment for 24 hrs (right). M7=MCF-7 cells alone; BST=breast fibroblasts alone; Co-c=MCF-7 cells cocultured with breast fibroblasts.

To determine if the stromal-epithelial signaling process requires that the fibroblasts be of normal origin as opposed to tumor-derived, we compared co-culture of MCF-7 cells on Matrigel with either normal breast fibroblasts (designated BST) or breast tumor fibroblasts (designated Bork). Treatment was with Ca^{2+} ionophore for 24 hours, and apoptosis was quantitated using the cell death detection ELIZA. As can be seen in Figure 2, significant induction of apoptosis occurs only when normal breast fibroblasts are used, suggesting altered signaling from tumor fibroblasts that is incapable of inducing apoptosis in the breast epithelial cells.

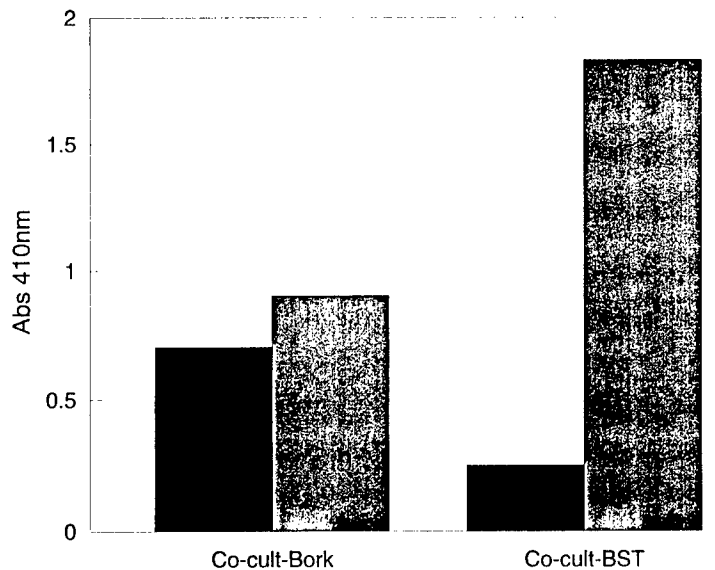


Figure 2. Induction of apoptosis in MCF-7 cells by normal vs. tumor breast fibroblasts. Each group of 2 consists of control untreated cells (left) and Ca^{2+} ionophore treatment for 24 hrs (right). Co-cult-Bork=breast tumor fibroblasts co-cultured with MCF-7 cells; Co-cult-BST=normal breast fibroblasts co-cultured with MCF-7 cells.

Elucidation of signals involved in the induction of programmed cell death (Task 2; see Appendix for copy of original Statement of Work)

To determine if cell-cell contact is necessary or if there is a secreted signal that results in programmed cell death, we cultured breast epithelial cells and fibroblasts in Transwell dishes that allow secreted substances to exchange between the cell types but do not allow direct cell-cell contact. In these experiments, MCF-7 cells were grown on inserts that were coated with Matrigel, and the lower chamber either contained no cells, or normal breast fibroblasts as "feeder" cells. As can be seen in Figure 3, after 24 hours treatment with Ca^{2+} ionophore and analysis by cell death detection ELIZA, apoptosis is preferentially induced in the MCF-7 cells that have been "fed" by normal breast fibroblasts as opposed to the MCF-7 cells cultured on Matrigel inserts in the absence of feeder fibroblasts. These results suggest that a secreted factor(s) is involved in controlling the induction of apoptosis, however this process may involve signals from both the fibroblasts and the epithelial cells.

To determine if the signal is provided by the fibroblasts, we assessed the ability of conditioned media from breast fibroblasts to induce apoptosis in MCF-7 cells. In these experiments, MCF-7 cells were grown on Matrigel (in the absence of fibroblasts) and were treated with conditioned media from breast fibroblasts (normal vs. tumor) that were themselves either treated with Ca^{2+} ionophore or untreated. We also asked whether the additional treatment of the MCF-7 cells with Ca^{2+} ionophore was necessary or had any further effect on apoptosis. As can

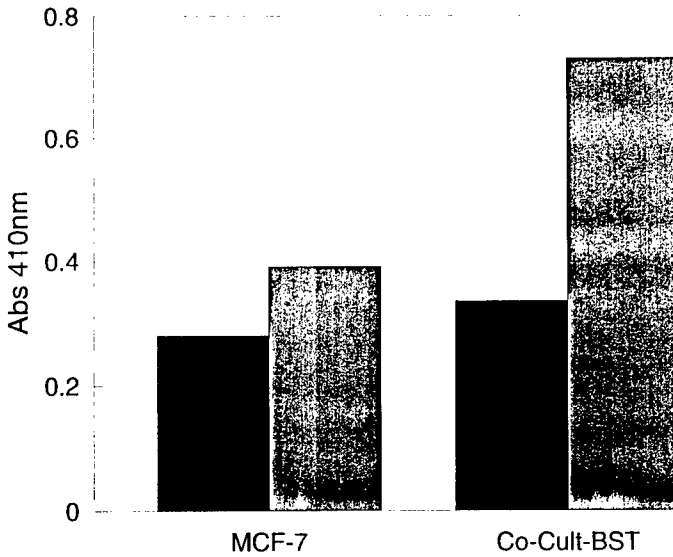


Figure 3. Induction of apoptosis in MCF-7 cells by "feeder" breast fibroblasts. Each group of 2 consists of control untreated cells (left) and Ca^{2+} ionophore treatment for 24 hrs (right). MCF-7=MCF-7 cells in the absence of feeder fibroblasts; Co-cult-BST= MCF-7 cells in the presence of feeder fibroblasts.

be seen in Figure 4, conditioned media from breast tumor fibroblasts was unable to induce apoptosis in MCF-7 cells, regardless of Ca^{2+} ionophore treatment, while conditioned media from normal breast fibroblasts was able to induce apoptosis, but only when the fibroblasts had been treated with Ca^{2+} ionophore. Treatment of the MCF-7 cells with Ca^{2+} ionophore had no further effect on apoptosis. Thus it appears that treatment of normal breast fibroblasts results in the production of a secreted signal that is sufficient to induce apoptosis in MCF-7 breast tumor cells. We have begun fractionating conditioned media in order to characterize this secreted factor(s), and have established to date that the signaling activity has a molecular weight of greater than 30kD.

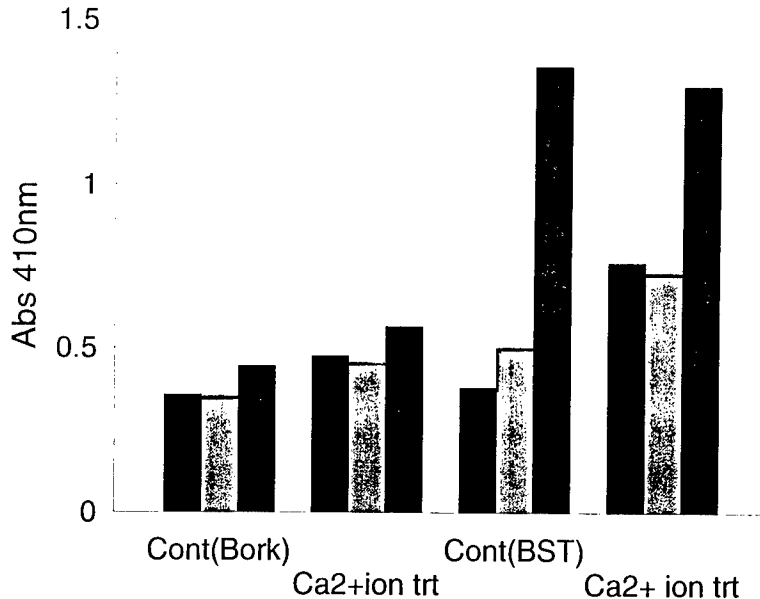


Figure 4. Induction of apoptosis in MCF-7 cells by conditioned media from breast fibroblasts. Each group of 3 consists of control MCF-7 cells (left), and MCF-7 cells treated with conditioned media from breast fibroblasts treated (right) or not (center) with Ca^{2+} ionophore for 24 hrs. Fibroblasts were either from tumor (Bork; left 2 groups) or normal tissue (BST; right 2 groups). Ca^{2+} ion trt = MCF-7 cells were also treated with Ca^{2+} ionophore (groups 2 and 4).

CONCLUSIONS

Our work thus far indicates that we have a potential model system for studying the induction of apoptosis in breast cancer cells in vitro. It appears that both stromal-epithelial signaling and the influence of basement membrane components are important for antiestrogens to induce apoptosis rather than cell cycle arrest, and that there is a secreted signal from normal fibroblasts that can induce apoptosis in MCF-7 cells. Our goal is to define these critical regulatory signals that commit a cell to the apoptosis pathway. It is hoped that this work will lead to the design of new therapies that can induce this process and result in the effective treatment of breast cancer, as well as provide a valuable new in vitro model system as an alternative to animal models for testing the cytotoxic effects of potential therapeutic agents.

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We are currently preparing a manuscript on this work, and have submitted two grant applications towards the goal of continuing this project.

PERSONNEL

Personnel receiving support from this project:

Susan A. Chrysogelos (PI)
Kate Strand (Research Assistant)
Erik Thompson (Consultant)

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APPENDIX

STATEMENT OF WORK

THE ROLE OF CELL-CELL SIGNALLING IN THE INDUCTION OF APOPTOSIS IN BREAST CANCER CELLS

The primary objective of this project is to develop an in vitro culture system that mimics the interactions leading to programmed cell death in vivo. This model system will then be used to investigate the intercellular signals responsible for mediating programmed cell death in breast cancer cells with the hope that new strategies can be developed to induce programmed cell death and exploit this normal cellular mechanism for limiting growth. Additionally, the development of this in vitro model system will also provide a valuable alternative to animal models for testing the cytotoxic effects of potential therapeutic agents.

Task 1. Determine the requirements for apoptosis to occur in vitro following estrogen withdrawal. (months 1-12)

This model system will be developed using co-culture of normal or tumor breast fibroblasts with MCF-7 breast cancer cells on plastic alone, Matrigel (basement membrane) or Vitrogen (collagen I). Apoptosis will be assessed by the appearance of DNA ladders, TRPM-2 expression, and TUNEL assays to identify individual apoptotic cells. (months 1-12)

Task 2. Using the in vitro model system developed in task 1, elucidate the signals that are responsible for the induction of programmed cell death in breast cancer cells.

- a. By culturing breast epithelial and stromal cells using Transwell dishes it will be determined if cell-cell contact is required for apoptosis to occur or if there is a secreted signal. (months 13-18)
- b. Conditioned media will be used to assess the role of a secreted factor. (months 19-24)



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Information Management

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