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14. ABSTRACT E-cadherin, is the major adhesion protein associated with epithelial malignancies and loss of E-cadherin expression is diagnostic of EMT in such cells. Loss of E-cadherin plays an important role in breast cancer progression, invasion and metastasis and is used as a prognostic marker for breast cancer. E-cadherin expression is significantly reduced in basal-like and triple negative breast cancers and a higher proportion of E-cadherin aberrations are observed in ER-α negative tumors. Interestingly, E-cadherin, has been shown to interact with ER and studies have demonstrated a direct role for ER-α in controlling E-cadherin expression, with elimination of ER-α from an ER-positive cell line or its reintroduction in an ER-negative context, respectively triggering repression or transcription of E-cadherin. Thus, ER-α may represent the prime factor controlling the expression of this gene in breast cancer cells, an idea previously suggested only by indirect evidence. In addition to a number of relational observations, the absence of ER-α has been mechanistically linked to E-cadherin suppression and EMT and indirect evidence suggests that re-expression of endogenous ER-α is linked to reversion of the invasive breast cancer phenotype. The connection between ER-α and E-cadherin, therefore, is complex and requires a detailed investigation. Several studies have associated loss of ER with worse tumor grade, aggressive biologic behavior and highly radioresistant breast cancer. However no studies have as yet clearly defined the role of E-cadherin in governing radiosensitivity and therefore, in this grant we propose to investigate this connection in greater detail.

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Introduction: Breast cancer is a heterogeneous disease that affects over one million women worldwide every year and includes a wide range of histologic and molecular subtypes that display diverse clinical behaviors. One such subgroup – the "triple negative" (i.e. estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative), includes breast cancers that have undergone an epithelial-to-mesenchymal transition (EMT), are associated with highly invasive clinical disease, have a higher mitotic index, and a worse clinical outcome. E-cadherin, is the major adhesion protein associated with epithelial malignancies and loss of E-cadherin expression is diagnostic of EMT in such cells. Loss of E-cadherin plays an important role in breast cancer progression, invasion and metastasis and is used as a prognostic marker for breast cancer. It has been found that EMT and E-cadherin expression are influenced by several growth factors that are responsive to signaling, as well as by a variety of polypeptide growth pathways. E-cadherin expression is significantly reduced in basal-like and triple negative breast cancers and a higher proportion of E-cadherin aberrations are observed in estrogen receptor-α (ER-α) negative tumors. Interestingly, E-cadherin, has been shown to interact with ER and studies have demonstrated a direct role for ER-α in controlling E-cadherin expression, with elimination of ER-α from an ER-positive cell line or its reintroduction in an ER-negative context, respectively triggering repression or transcription of E-cadherin. Thus, ER-α may represent the prime factor controlling the expression of this gene in breast cancer cells, an idea previously suggested only by indirect evidence. In addition to a number of relational observations, the absence of ER-α has been mechanistically linked to E-cadherin suppression and EMT and indirect evidence suggests that re-expression of endogenous ER-α is linked to reversion of the invasive breast cancer phenotype. The connection between ER-α and E-cadherin, therefore, is complex and requires a detailed investigation. Several studies have associated loss of ER in breast cancer with worse tumor grade and aggressive biologic behavior. Furthermore, lack of ER-α correlates with highly radioresistant breast cancer. However no studies have as yet clearly defined the role of E-cadherin in governing radiosensitivity and therefore, in this grant we propose to investigate this connection in greater detail. It is also interesting to note that the triple-negative breast cancer patients who develop a local-regional recurrence have a very poor prognosis, indicating that identification of new therapies with activity in the triple negative subtype of breast cancer is a clinical priority.

Hypothesis/Rationale/Purpose: The central hypothesis being tested in this project is that EMT determines the radiosensitivity of breast cancer cells. We propose studies to investigate the impact of ER-α and E-cadherin radiation resistance of breast tumors as well as their role in mediating EMT.

Objectives: Our grant has the following two specific aims:
Aim #1: Does EMT govern the radiosensitivity of breast cancer cells? To understand the functional relationship of ER-α and E-cadherin with respect to mediating EMT and governing radiation response of breast cancer cells? **Aim #2:** Which cell signaling pathway downstream of E-cadherin and ER-α mediates the radioresponse of breast tumor cells?

The subaims under **Aim #1** are listed below:

i). Compare the basal levels of proteins involved in the EMT process including E-cadherin, Vimentin, Snail, Zeb-1 and Zeb-2, Twist, and Slug in a panel of ER-negative and ER-positive human breast cancer cell lines (MDA-MB-231, MCF-7, Hs578t, MDA-MB-468) by Western Blot Analysis.

ii). Compare the intrinsic radiosensitivity of the panel of breast cancer cell lines listed above, using clonogenic cell survival assay.

iii). Test whether re-expressing and stably transfecting breast cancer cells with a plasmid over-expressing E-cadherin in two breast cancer lines, MDA-MB-231 and MDA-MB-468 cells, sensitizes them to radiation. For this we will use a *CDH1* (E-cadherin gene) expression vector from OriGene Technologies Inc. This vector has the cDNA for *CDH1* inserted into the pCMV6-neo vector and has been specifically designed by the company for the purpose of making stable clones. We will transfect the above mentioned cell lines with this vector using FuGENE 6 transfection reagent. Stable clones will be selected using neomycin (G418). For the control vector, we will excise out the *CDH1* gene and use the religated backbone vector to prepare cells stably expressing the control vector. The ability of E-cadherin to radiosensitize cells upon restoration into the cell lines will be evaluated using clonogenic cell survival assays.

We will also utilize the tetracycline-inducible promoter system to clone E-cadherin. The basic system will be obtained from Clontech. Briefly, we will transfect MDA-MB-231 cells with the pTet-On-Advanced vector and select G418 resistant cells. We will insert the cDNA for *CDH1* into the pTRE-Tight vector and transfected the Tet-On-Advanced MDA-MB-231 cells with the pTRE-Tight-CDH1 response plasmid and the linear hygromycin marker. Hygromycin-resistant cells will be selected and cloned out. Clones will be treated with various doses of doxycycline and tested for E-cadherin expression by western blot. We will extend this approach to test the other E-cadherin negative cell line, MDA-MB-468, in order to extend this to other non-E-cadherin expressing breast cancer lines, i.e. that they are radiosensitized when E-cadherin expression is restored. In each case, parental cells without vector and parental cells containing only the pTet-On-Advanced vector, will be treated with doxycycline to ensure that doxycycline does not affect radiosensitivity. Set up clonogenic cell survival assays to assess radiosensitivity of the above mentioned cell lines.
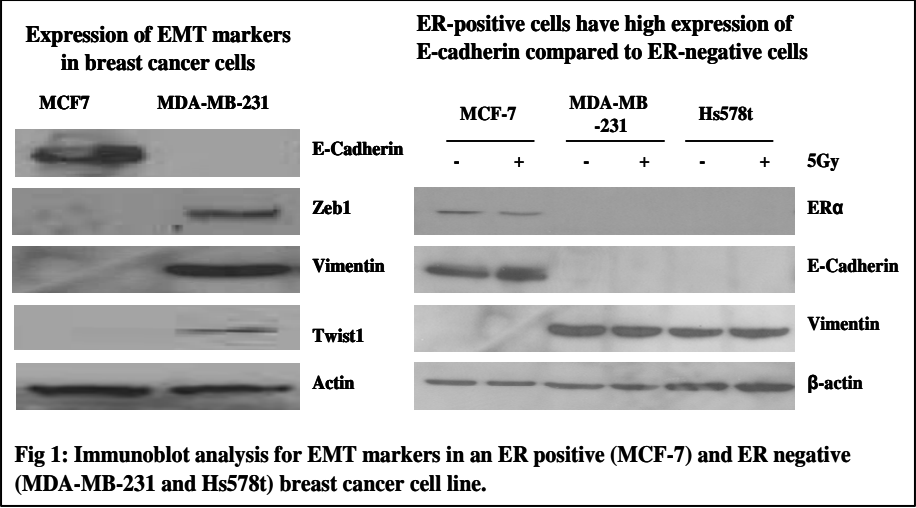
iv). Test whether knocking down E-cadherin in E-cadherin expressing breast cancer cell lines makes them radioresistant. We will use siRNA approach to downregulate E-cadherin in breast cancer cells and associate downregulation of E-cadherin to radiosensitivity.

We will use siRNA to *CDH1* from Ambion and transfect MCF-7 cells TransIT-TKO transfection reagent. Immunoblot analysis of the transfected cells will be carried out to check for lowered expression of E-cadherin compared to controls over time periods of 1-4 days. Control siRNA, also obtained from Ambion will be used as the control. These preliminary experiments will establish proof-of-principle, i.e. knocking down E-cadherin expression in E-cadherin expressing cells induces radioresistance. The problem with this approach, however, is that the knockdown is only transient, i.e. 3 days, and 3 days may not be sufficient to maximally alter the pathways that mediate radioresistance. As an alternative approach, we will use shRNA expression vectors from OriGene that target *CDH1*. We will isolate stable clones that show reduced expression of E-cadherin. Non-targeting shRNA will also be obtained from OriGene and used as a negative control. Immunoblot analysis to check for suppressed expression of E-cadherin in clones of MCF-7 will be carried out.

Key Research Accomplishments
The progress made towards each sub-specific aim under **Aim#1** is briefly summarized in this section.
As a first test of our hypothesis we examined a panel of human breast cancer cell lines for estrogen receptor-α and E-cadherin expression by western blot analysis. The panel included ER-α positive (MCF-7) and ER-α negative (MDA-MB231 and Hs578t) cell lines. All three cell lines were examined
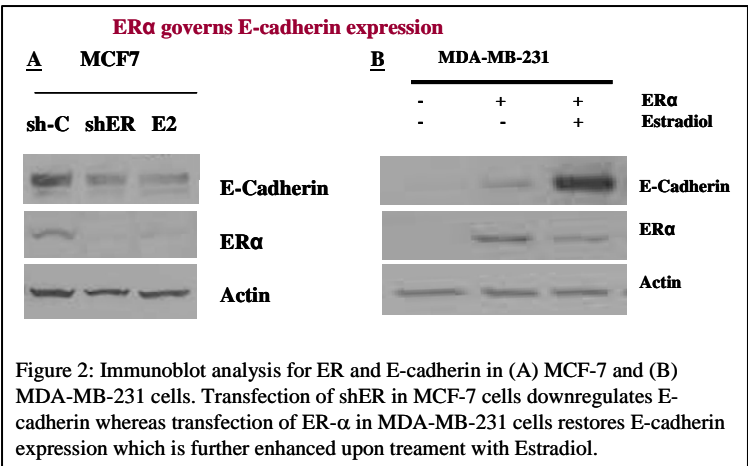
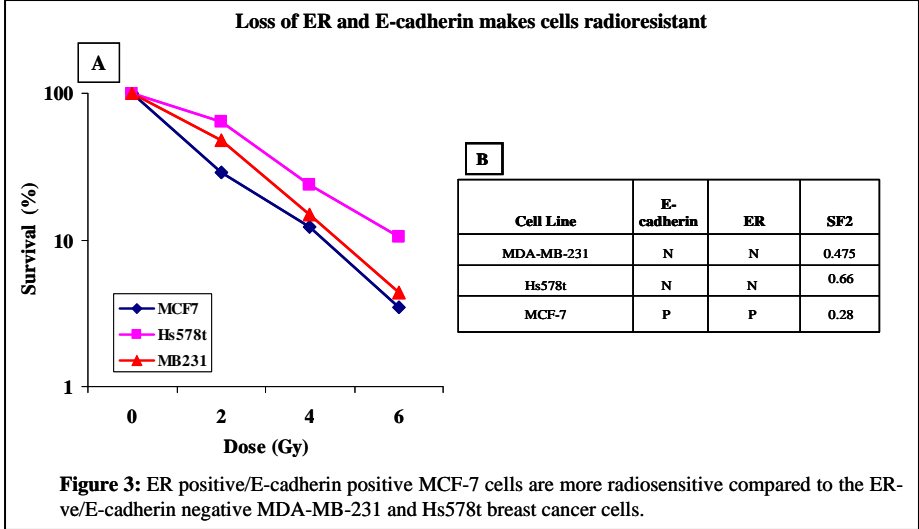for E-cadherin (epithelial marker), ER-α and Vimentin (mesenchymal marker) expression. As can be seen from Figure 1, ER-α negative cell lines demonstrated a loss of E-cadherin but a gain of vimentin compared to the ER-α positive MCF-7 cell line.

Fig 1: Immunoblot analysis for EMT markers in an ER positive (MCF-7) and ER negative (MDA-MB-231 and Hs578t) breast cancer cell line.

To further correlate the loss of ER with E-cadherin we used MCF-7 cells stably expressing shRNA to ER-α. These cells were analyzed for estrogen receptor expression as well as for E-cadherin. As can be seen from Figure 2A MCF-7 sh-Control cells had high levels of expression of E-cadherin as well as ER-α. Knockdown of ER expression resulted in a loss of E-cadherin as well. Addition of Estradiol (E2) did not alter the level of ER-α or E-cadherin. Similarly, transient transfection of MDA-MB-231 cells with ER-α, not only led to expression of ER-α but also led to expression of E-cadherin as detected by Western blot analysis (Figure 2B). Treatment of these cells with Estradiol led to a further increase in E-cadherin indicating the estrogen receptor governs E-cadherin expression. This observation will however need to be examined in greater detail.

Figure 2: Immunoblot analysis for ER and E-cadherin in (A) MCF-7 and (B) MDA-MB-231 cells. Transfection of shER in MCF-7 cells downregulates E-cadherin whereas transfection of ER-α in MDA-MB-231 cells restores E-cadherin expression which is further enhanced upon treament with Estradiol.

The intrinsic radiosensitivity of these breast cancer cell lines (MCF-7, Hs578t, MDA-MB-231) was compared using clonogenic cell survival assay. As shown in Figure 3, cell lines expressing estrogen receptor (MCF-7) were more sensitive to increasing doses of radiation when compared with the ER negative cells (MDA-MB-231, and Hs578t). A comparison of the survival fraction at 2Gy (SF2) for these cell lines is shown in Figure 3B. ER-α negative and E-cadherin

Cell Line	E-cadherin	ER	SF2
MDA-MB-231	N	N	0.475
Hs578t	N	N	0.66
MCF-7	P	P	0.28

Figure 3: ER positive/E-cadherin positive MCF-7 cells are more radiosensitive compared to the ER-ve/E-cadherin negative MDA-MB-231 and Hs578t breast cancer cells.

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negative cell lines had higher SF2 values when compared with the ER-α positive-E-cadherin positive MCF-7 cells indicating intrinsic radioresistance of ER-ve/E-cadherin negative cells.

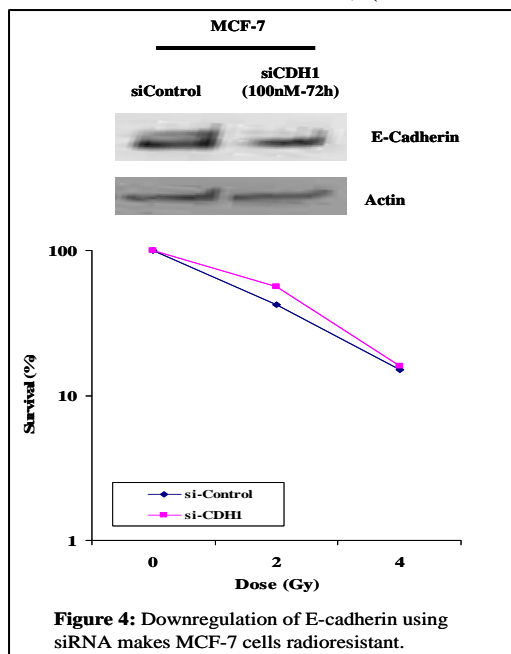Since MCF-7 cells, (ER and E-cadherin positive) were the most radiosensitive among the 3 cell lines we decided to test the role of E-cadherin (CDH1) in mediating radiosensitivity. For that purpose MCF-7 cells were transiently transfected with siCDH1. Studies were conducted to optimize the dose and time of treatment with the siRNA to CDH1. We found that treatment of cells with 100nM dose over a period of 72 hrs showed maximal downregulation of E-cadherin as detected by Western blot anlaysis (Figure 4). MCF-7 cells transfected with siRNA-Control were used as controls. The siCDH1 and siCONT cells were compared for their radiosensitivity in a clonogenic cell survival assay following exposure to various doses of radiation. As shown in Figure 4, siCDH1 transfected MCF-7 cells demonstrated an increased resistance to radiation when compared with the vector control cells. These experiments however need to be repeated at least three times to ensure reproducibility and obtain statistically significant data.

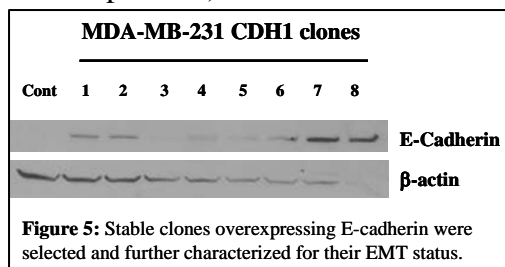**Figure 4:** Downregulation of E-cadherin using siRNA makes MCF-7 cells radioresistant.

Figure 5: Stable clones overexpressing E-cadherin were selected and further characterized for their EMT status.

To further test the loss of E-cadherin with radiation resistance we prepared MDA-MB-231 stable cells overexpressing E-cadherin. Several clones overexpressing E-cadherin were selected and analyzed by western blot analysis (Figure 5)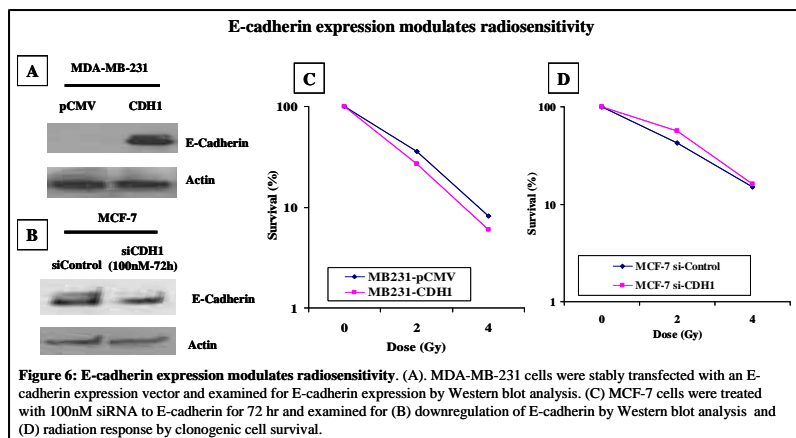. These clones were further expanded and analyzed by western blot analysis for expression of EMT related markers and for sensitivity to radiation. We picked clone #7 for all further experiments because of a nice correlation observed between E-cadherin overexpression and radiation response. Clonogenic cell survival experiments were set up to compare the radiation response of MDA-MB-231-CMV control cells versus MDA-MB-231-CDH1 clone 7. Overexpression of E-cadherin made the MB-231 cells sensitive to radiation as can be seen in Figure 6. We have obtained similar results using Hs578t cells stably expressing E-cadherin.

Figure 6: E-cadherin expression modulates radiosensitivity. (A). MDA-MB-231 cells were stably transfected with an E-cadherin expression vector and examined for E-cadherin expression by Western blot analysis. (C) MCF-7 cells were treated with 100nM siRNA to E-cadherin for 72 hr and examined for (B) downregulation of E-cadherin by Western blot analysis and (D) radiation response by clonogenic cell survival.

Work on Aim #2 is currently ongoing and will be reported in the next cycle.