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<p>Loss of estrogen receptor (ER) function has been associated with hyperactive ERK1/2, which culminates in aggressive, radiation resistant cancers. The ERK1/2 pathway has also been linked to DNA damage and repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling. An increased DNA repair capacity in ER-α negative breast tumors has been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER-α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins. We compared ER-α positive and negative cells for expression levels of ERK1/2 and DNA repair proteins involved in the repair of radiation-induced double strand breaks. Preliminary data obtained from clonogenic cell survival assays showed that ER-α positive cells were more radiosensitive compared with the negative cells. These cell lines are also being compared for the expression of ERK1/2 and its downstream proteins and proteins involved in repair by Western blot analysis. We are also evaluating the ability of inhibitors of the ERK1/2 pathway to restore radiosensitivity to the ER negative cell lines. The effect of these inhibitors on expression of DNA repair proteins and their ability to restore ER-α expression will also be tested. The outcome of these studies will have a potential impact in the clinic and benefit breast cancer patients</p>						
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**U. T. M.D. Anderson Cancer Center
Department of Experimental Radiation Oncology
Final Report July, 2011**

Report on Breast Cancer Idea Award

**Estrogen receptor/MAPK cross-talk as a mechanism of radiation resistance of
breast cancer**

Principal Investigator: Dr. Anupama Munshi

Study Site:
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Report Date: July 2011

Introduction

Breast cancer is the most commonly occurring cancer among women (22% of all cancers in 2000) and is second only to lung cancer as a cause of cancer deaths in women (15% of cancer deaths) (1, 2). The estimated annual incidence of breast cancer worldwide is about one million cases with ~200,000 cases in United States (27% of all cancers in women) and ~320,000 cases in Europe (31% of all cancers in women) (3, 4). Over the last two decades, the annual incidence rate in US has been increasing steadily (5). Women with an early diagnosis and favorable risk factors are cured by primary surgical and radiotherapy treatment while those with more advanced or aggressive tumors experience recurrence and later death (5). Risk factors for recurrence are generally related directly or indirectly to the rate of cell proliferation and the percentage of cells undergoing apoptosis. The factors controlling these two interrelated processes are complex and not fully understood.

Radiotherapy of patients with breast cancer remains an important cancer treatment modality and plays an essential role in local and regional control of the disease (6). It has been estimated that more than 50% of all cancer patients receive radiation as part of their overall management. Randomized trials have demonstrated the efficacy of radiation therapy in the treatment of breast cancer. Even though many of these patients benefit from their treatment, between 30-50% of patients with localized disease initially fail at their primary tumor sites following therapy. A variety of strategies have been and are continuing to be actively explored to improve local control. Tumors locally fail after radiation therapy due to biological factors associated with the particular tumor. Advances in our knowledge of the molecular pathways that govern some of these factors has generated many new ideas that can be explored for improving the efficacy of radiation therapy but there are still aspects of tumor sensitivity to radiation that are poorly understood (7-9).

Since radiation therapy plays a critical role in the management of a majority of breast cancer patients, identification of factors that help predict which patients are at risk for relapse within the irradiated field remains an active area of investigation. A substantial amount of research has been devoted to identifying predictive markers for radiation resistance. Loss of estrogen receptor (ER) function has been associated with constitutive and hyperactive MAPK (particularly ERK1/2), which culminates in aggressive, metastatic, radiation-resistant cancers. Activation of the ERK1/2 cascade modulates the phosphorylation and activity of several nuclear transcription factors that in turn regulate a series of genes involved in promoting cellular survival and resistance to chemotherapy and ionizing radiation. The ERK1/2 pathway has also been linked to DNA damage and DNA repair, with multiple proteins involved in DNA repair being transcriptionally regulated through ERK1/2-dependent signaling (10-21). An important hallmark that dictates the radioresistant phenotype of tumor cells and is probably the most critical factor in the radiation responsiveness of a tumor is the ability of a cancer cell to repair and recover from radiation-induced DNA double-strand breaks (DSBs). An increased DNA repair capacity in ER- α negative breast tumors has also been implicated as a mechanism of radioresistance. We postulate that the mechanism of development of radiation resistance in the ER- α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

Body

Breast cancer is a heterogeneous disease, displaying wide variances in response to various therapeutic approaches and outcome. Generally, hormone receptor negative tumors are high grade, poorly differentiated tumors. In accordance with these observations, decreased survival rates are reported for patients with estrogen- or progesterone-receptor negative tumors compared to those with hormone receptor positive breast cancer (22, 23).

The epidermal growth factor receptor (EGFR)/Her-2/neu/Ras/MEK/mitogen activated protein kinase (MAPK) and the c-kit-Akt / PI3K (phosphoinositol-3-kinase) pathways are two major signal transduction pathways that lead to activation of intracellular driving mechanisms for proliferation and antiapoptotic features of tumor cells. It has been previously demonstrated that MAPK family members, including ERK, JNK and p38 MAPK play an active role in the proliferation, invasive capacity and generation of metastatic potential for cancer cells, as well as chemoresistance (10-21). Furthermore, the MAPK family has been shown to have a regulatory role in providing the complex balance between cellular growth and death through competing interactions. Therefore, the exact mechanism by which MAPK is involved in the pathogenesis of breast cancer is not clear and remains to be elucidated further.

Intracellular signaling through the Ras-MAPK pathway has been observed in a wide range of breast tumors and has been linked to non-genomic estrogen-mediated tumor growth and induction of estrogen receptor-negative phenotype, in addition to resistance to hormonal agents, such as tamoxifen (24-33). MAPK overexpression has also been associated with growth factor related and anchorage-independent tumor proliferation by increased heat shock protein expression in triple negative tumors and is in concordance with in vitro data suggesting that active MAPK signaling is correlated with estrogen receptor negativity and induction of receptor negative phenotype (24-33). The role of MAPK has not been extensively evaluated in a prospective trial, and data available is generally limited to analysis of archival material.

We postulate that the mechanism of development of radiation resistance in the ER- α negative breast cancer cells involves a dynamic interplay between the ERK1/2 pathway and DNA repair proteins.

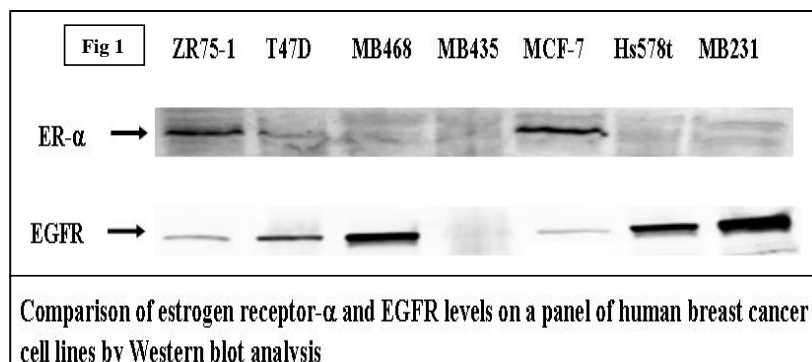
Aim 1: Test the hypothesis that ER- α negative breast cancer cells have hyperactive ERK signaling and an enhanced DNA repair capacity that contributes to radiation resistance

- i). Compare the basal levels of activated ERK1/2 and levels of DNA repair proteins (BRCA1, BRCA2, DNA-PK, NBS1, RAD51, Gadd-45 and Topo-II α) in ER- α negative (MDA-MB-231 and Hs578t) and ER- α positive (MCF-7 and T47D) breast cancer cell lines by Western Blot Analysis.
- ii). Construct MCF-7 cells (ER- α positive), stably transfected with an expression vector carrying activated ERK1/2 under an inducible promoter to serve as a model system to address the role of ERK1/2 in mediating a loss of ER- α expression and leading to radioresistance.
- iii). Use siRNA approach to downregulate ER- α in MCF-7 cells and associate loss of ER- α to hyperactivation of ERK1/2 and DNA repair proteins.
- iv). Carry out Host Cell Reactivation and Comet Assays to determine the intrinsic DNA repair capacity and the capacity to repair radiation-induced DNA double strand breaks in the cell lines mentioned above.
- iv). Set up clonogenic cell survival assays to assess radiosensitivity of the above mentioned cell lines.

Key Research Accomplishments

The progress made towards each sub-specific aim is briefly summarized in this section.

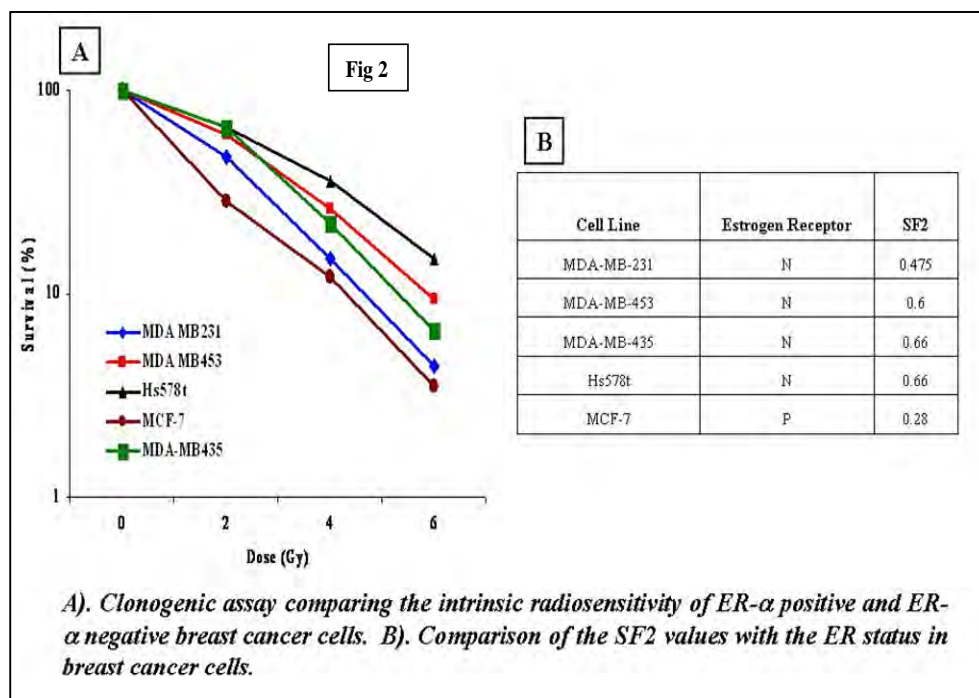
As a first test of our hypothesis we examined a panel of human breast cancer cell lines for



estrogen receptor-α expression by western blot analysis. The panel included ER-α positive (MCF-7, ZR75-1 and T47D) and ER-α negative (MDA-MB231, MDA-MB468 and MDA-MB-435) cell lines. Since over-expression of EGFR is inversely correlated with ER-α we also looked for EGFR expression in the cell

lines mentioned above by Western blot analysis. ER-α negative cell lines had high expression of EGFR compared to the ER-α positive cells (Figure1).

Once the ER-α status in these cell lines was confirmed we compared the intrinsic radiosensitivity of some of these breast cancer cell lines using clonogenic cell survival assay. As

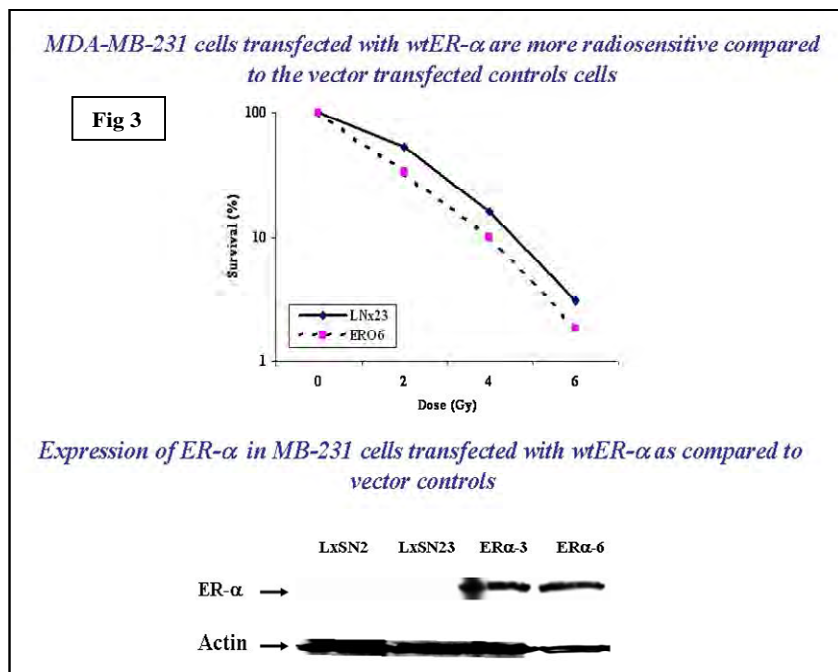


shown in Fig2A, the cell lines expressing estrogen receptor (MCF-7) were more sensitive to increasing doses of radiation when compared with the ER negative cells (MDA-MB-231, MDA-MB-453, MDA-MB-435 and Hs578t). A comparison of the survival fraction at 2Gy (SF2) of these cell lines is shown in Fig 2B.

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

In addition we tested MDA-MB-231 cells that were stably transfected with full length ER-α (clones designated ERα-3 and ERα-6). MB231 cells transfected with vector backbone were used as controls (designated LxSN2 and LxSN23). The generation of these stable cell lines has been described in detail by Dr. Nakshatri from whom the cells were obtained LxSN23 and ERα-6 cells were compared for their radiosensitivity in a clonogenic cell survival assay

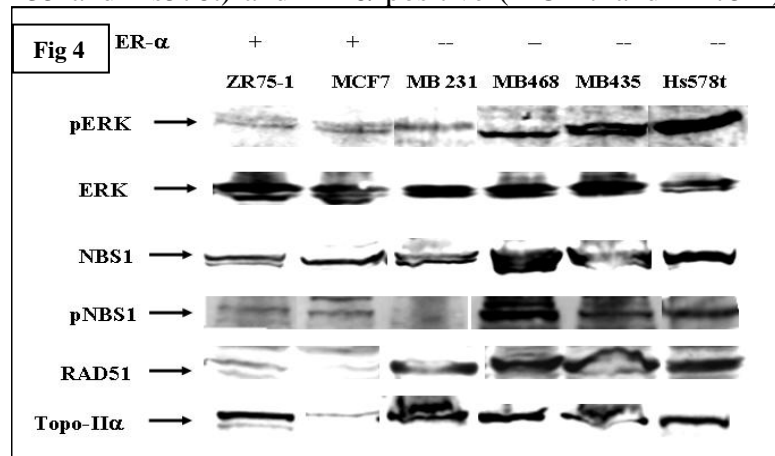
following exposure to various doses of radiation. As shown in Fig 3A the estrogen receptor expressing ER α -6 clone was more sensitive to increasing doses of radiation when compared with the vector control cells.



the vector control cells. The survival enhancement ratio was enhanced when the estrogen receptor gene was put back into the cells. Both the cell lines were also compared for the level expression of ER- α by western blot analysis.

Fig 3B clearly demonstrates the lack of ER- α protein in the vector control cells and a robust expression in the full length ER- α transfected MB231 cells. Following these experiments, we compared the basal levels of activated ERK1/2 and levels of DNA repair proteins

(NBS1, RAD51, and Topo-II α) in ER- α negative (MDA-MB-231, MDA-MB-468, MDA-MB-435 and Hs578t) and ER- α positive (MCF-7 and ZR75-1) breast cancer cell lines by Western

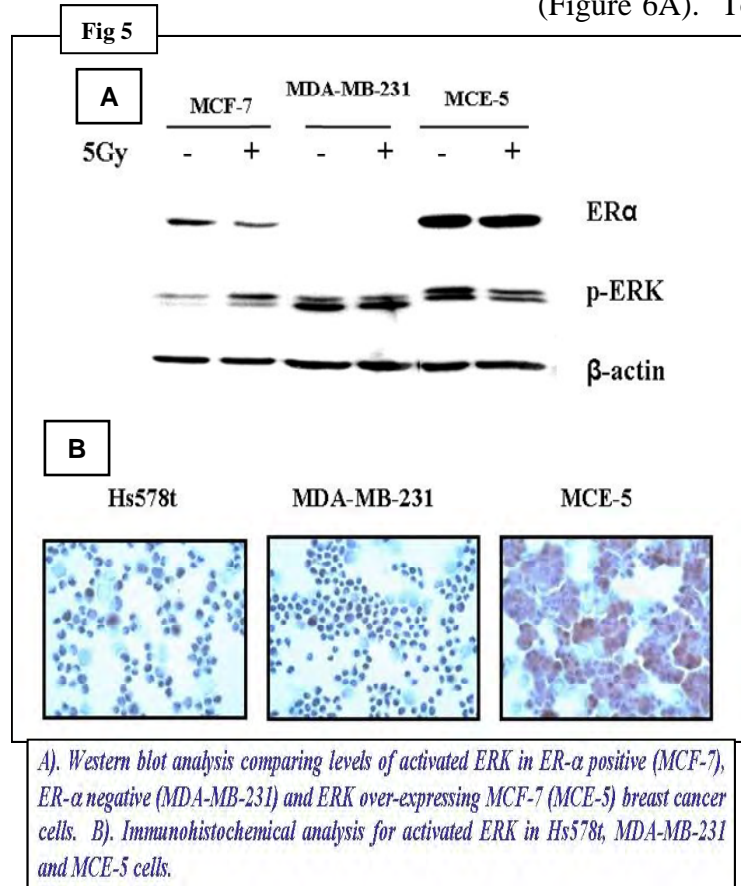


Blot Analysis. As can be seen in Fig 4 ER- α negative cells have higher levels of phosphorylated ERK and DNA repair proteins such as phospho-NBS1 and RAD51. Levels of Topo-II α were also higher in ER- α negative breast cancer cell lines. However ZR75-1, an ER- α positive cell line, also expressed high levels of Topo-II α .

Since transient/constitutive expression of MAPK leads to

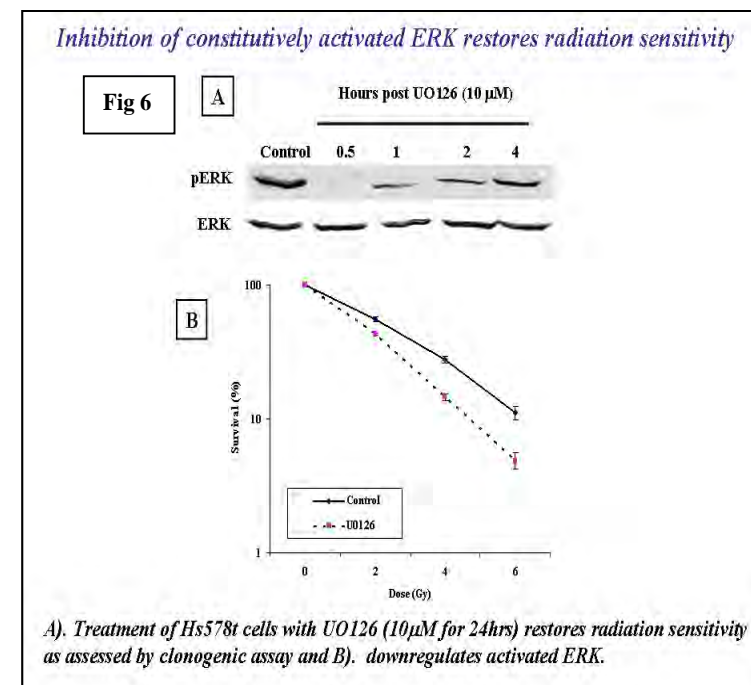
downregulation of ER- α we obtained an MCF-7 breast cancer clone engineered to overexpress EGFR and thereby activated phospho-MAPK/ERK. This cell line, designated as MCE-5, was obtained from Dr. Dorraya El-Ashry (University of Michigan, Ann Arbor, MI). We compared the levels of pERK and ER- α in MCE-5 and MDA-MB-231 cells. As shown in Fig 5A the MCE-5 cells had a higher constitutive level of pERK when compared to MCF-7 cells. Exposure to 5Gy dose of radiation led to an increase in ERK levels in the MCF-7 cells but not in the MCE-5 or the MDA-MB-231 cells. Immunohistochemical analysis was also performed on the Hs578t, MDA-MB-231 and the MCE-5 cells for activated ERK. As shown in Fig 5B, MDA-MB-231 and Hs578t cells showed positive staining for ERK. The MCE-5 cells overexpressing activated ERK however were very strongly positive for ERK by immunohistochemistry.

As shown in the figures above ERK is constitutively active in Hs578t and MDA-MB-468 cells as detected based on phospho-p44/p42 expression. Therefore, we used the ERK inhibitor UO126 to test whether the MEK/ERK pathway was playing a role in radiation resistance of these ER- α negative cells. Treatment of Hs578t cells with 10 μ M dose of UO126 for as little as 30 min down-regulated phospho-ERK and this inhibition was sustained for up to 24 hrs of treatment (Figure 6A). To determine the ability of UO126 to act



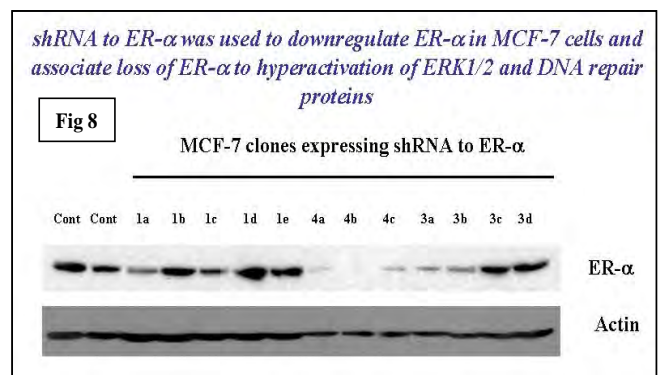
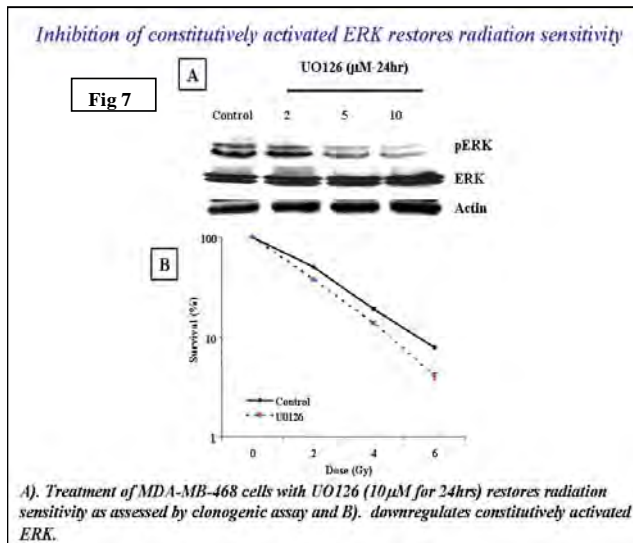
as a radiosensitizer, Hs578t cells were exposed to 10 μ M UO126 for 24 hrs, irradiated and harvested for clonogenic assay. UO126 restored radiation sensitivity to Hs578t, ER- α negative cells, which are known to be extremely radioresistant (Figure 6B). It will be important to see if UO126 can suppress both constitutive and radiation-induced ERK activation as well as examine its ability to restore radiation sensitivity following short treatments such as 30 minutes.

Similar results were obtained with MDA-MB-468 cells (Figure 7A and 7B). Cells were treated with varying doses of UO126- 2, 5 and 10 μ M for a period of 24 hrs. Treatment with both 5 μ M and 10 μ M dose of UO126 suppressed phosphorylated ERK1/2 levels. Treatment with 10 μ M dose of UO126



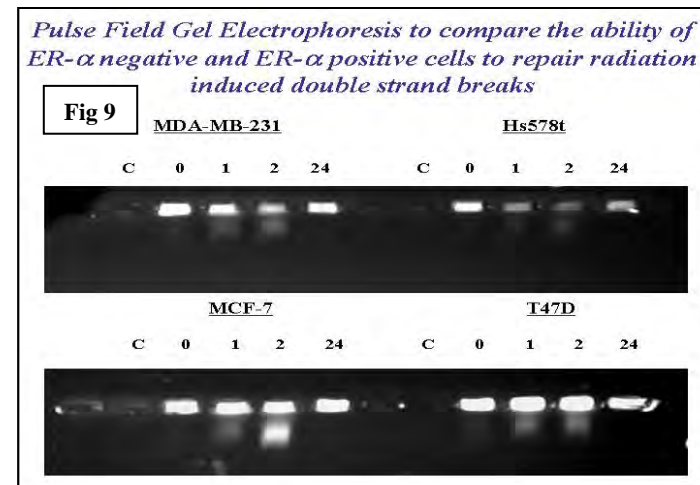
also restored radiation sensitivity as assessed by clonogenic cell survival experiments.

In addition we also prepared an MCF-7 clone in which ER- α levels were knocked down using shRNA to ER- α . MCF-7 cells which are ER- α positive, were stably transfected with shRNA to ER- α and as shown in the figure (Figure 8) below, several clones showing downregulation of ER- α were selected and analyzed by western blot analysis. These clones were expanded and frozen for future use to associate loss of ER- α to hyperactivation of ERK1/2 and DNA repair proteins.



Pulse Field Gel Electrophoresis was done as described previously. Briefly, cells were irradiated on ice with 40 Gy. Immediately

after irradiation, the medium was replaced with warm medium and the cells were placed in a 37°C incubator for the appropriate time for repair. Cells were then trypsinized on ice, washed, and embedded in agarose plugs. The plugs were lysed and digested with proteinase K. DNA fragments were separated using a CHEF-DR III system (Bio-Rad Laboratories) at 1.5 V/cm for 20 h at 25°C in 0.5x Tris-borate EDTA buffer. After electrophoresis, the gel was transferred to a nylon membrane for 3



days at room temperature. The membrane was then hybridized with a ^{32}P -labeled human Alu+ probe for 18 h at 45°C. The fraction of DNA released into the lane and that remaining in the plug was determined on the membrane using a Typhoon 9400 storage phosphorimaging system and ImageQuant software (Amersham Biosciences/GE Healthcare). We also analysed (shown in Fig 9) the intrinsic DNA repair capacity of ER-positive and ER-negative breast cancer cells as shown above. As can be seen MDA-MB-231 and hs578t cells had a greater capacity to repair DNA.

Aim 2: Determine combinations of targeted therapeutics that will effectively restore sensitivity to ionizing radiation.

- i). We will evaluate the ability of small molecule inhibitors (CI-1033 and UO126 to inhibit phosphorylated ERK1/2) to block constitutively activated ERK and restore radiosensitivity to the cell lines mentioned in Aim 1. Radiosensitization, determined on the basis of clonogenic survival, will be the critical endpoint of this series of experiments. (Months 14-20).
- ii). We will test the effect of these inhibitors on inhibition of activated ERK, expression of DNA repair proteins and their ability to restore ER- α expression suggesting the presence of a feedback mechanism in modulating ER- α expression. We will use Western blot analysis to examine relative protein levels prior to and after treatment with these inhibitors. Any

changes in the DNA repair capacity following treatment with the inhibitors will be assessed using the Comet and the Host cell reactivation assays. (Months 18-24).

ii). Construct MB231 cells (ER- α positive), stably transfected with an expression vector carrying activated ERK1/2 under an inducible promoter to serve as a model system to address the role of ERK1/2 in mediating a loss of ER- α expression and leading to radioresistance.

iii). Use siRNA approach to downregulate ER- α in MCF-7 cells and associate loss of ER- α to hyperactivation of ERK1/2 and DNA repair proteins.

iv). Carry out Host Cell Reactivation and Comet Assays to determine the intrinsic DNA repair capacity and the capacity to repair radiation-induced DNA double strand breaks in the cell lines mentioned above.

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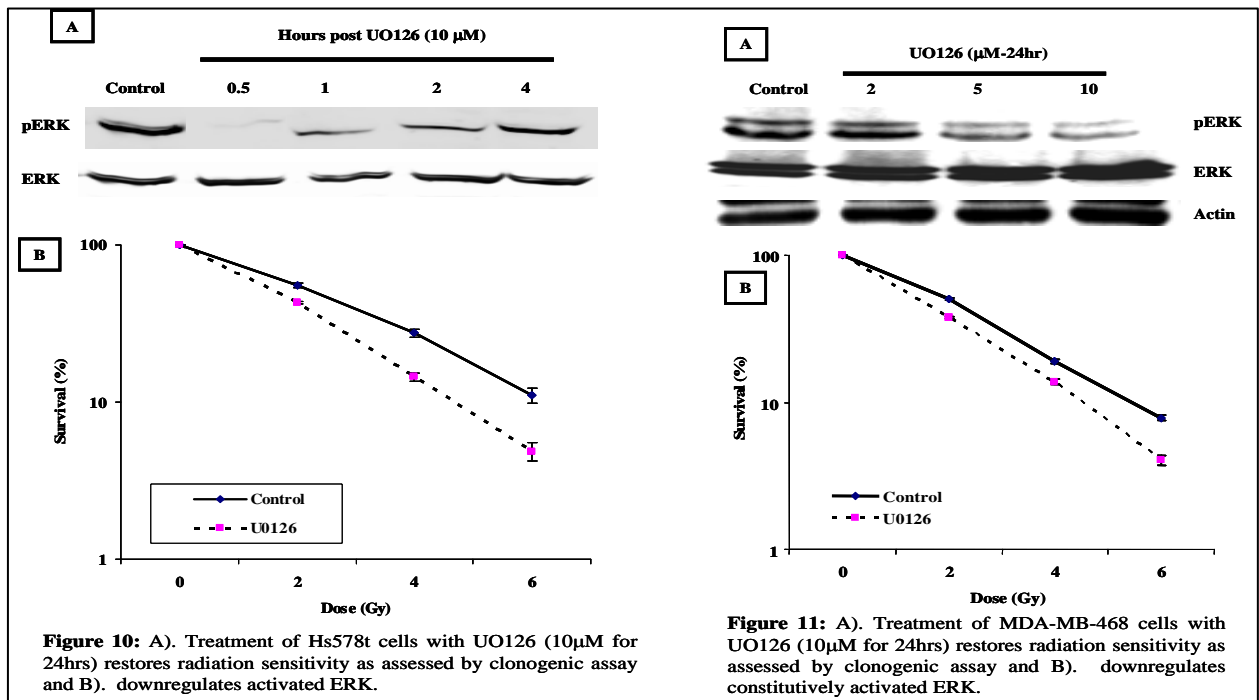
Aim 3: Generation of tissue arrays and immunohistochemical analysis of patient specimens for expression of DNA repair proteins and signaling intermediates in the ERK pathway.

i). We will evaluate the prevalence of the ERK pathway and its downstream targets, as well as DNA repair proteins (BRCA1, BRCA2, DNA-PK, GADD-45 and Topo-II α) in a cohort of clinical breast cancer specimens previously used to investigate for markers of locoregional failure after radiation therapy. An attempt will be made to correlate loss of ER- α with hyperactive ERK1/2 and high levels of DNA repair proteins in clinical samples. The samples will be analyzed by tissue microarray. (Months 24-36).

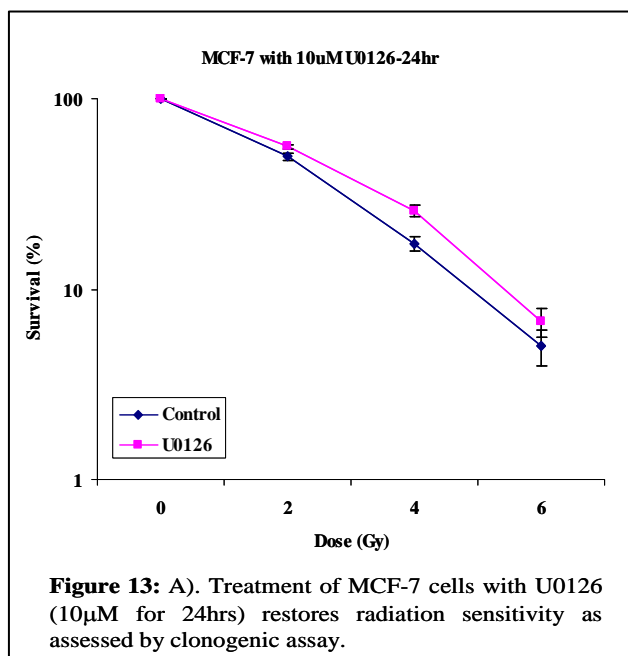
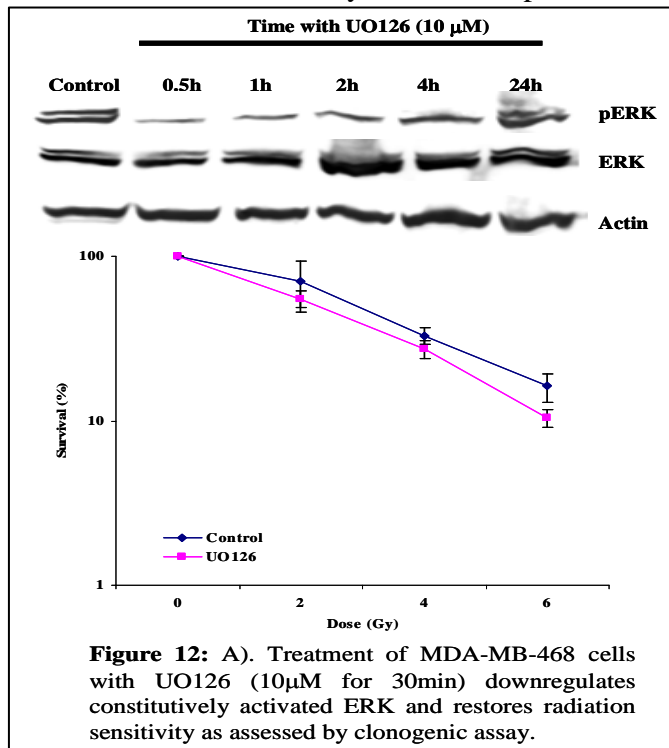
Aim#2

The progress made towards each sub-specific aim is briefly summarized in this section.

Since transient/constitutive expression of MAPK leads to downregulation of ER- α we obtained an MCF-7 breast cancer clone engineered to overexpress EGFR and thereby activated phospho-MAPK/ERK. This cell line, designated as MCE-5, was obtained from Dr. Dorraya El-

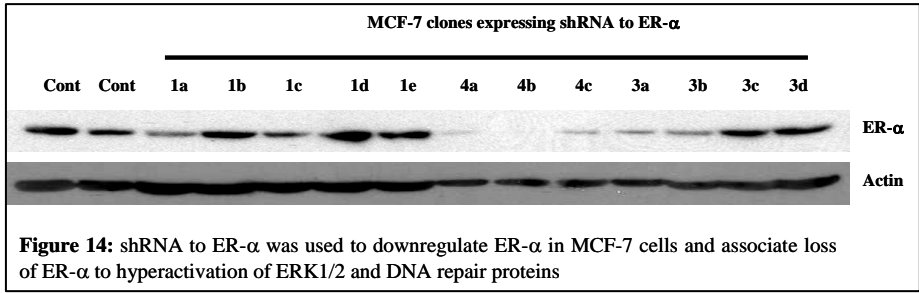


Ashry (University of Michigan, Ann Arbor, MI). We compared the levels of pERK and ER- α in MCE-5 and MDA-MB-231 cells. The MCE-5 cells had a higher constitutive level of pERK when compared to MCF-7 cells. Exposure to 5Gy dose of radiation led to an increase in ERK levels in the MCF-7 cells but not in the MCE-5 or the MDA-MB-231 cells. Immunohistochemical analysis was also performed on the Hs578t, MDA-MB-231 and the MCE-5 cells for activated ERK. MDA-MB-231 and Hs578t cells showed positive staining for ERK. The MCE-5 cells overexpressing activated ERK however were very strongly positive for ERK by immunohistochemistry.

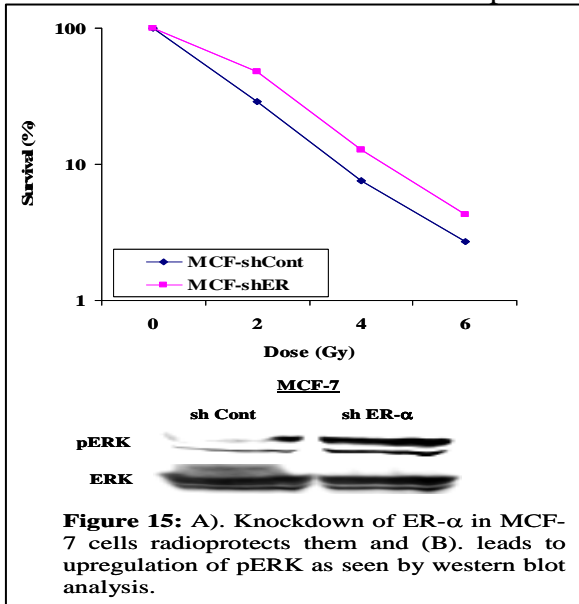


Since ERK is constitutively active in Hs578t and MDA-MB-468 cells as detected based on phospho-p44/p42 expression, we used the ERK inhibitor UO126 to test whether the MEK/ERK pathway was playing a role in radiation resistance of these ER- α negative cells. Treatment of Hs578t cells with 10 μ M dose of UO126 down-regulated pERK and this inhibition was sustained for up to 24 hrs of treatment (Figure 10A). To determine the ability of UO126 to act as a radiosensitizer, Hs578t cells were exposed to 10 μ M UO126 for 24 hrs, irradiated and harvested for clonogenic assay. UO126 restored radiation sensitivity to Hs578t, ER- α negative cells, which are known to be extremely radioresistant (Figure 10B). Similar results were obtained with MDA-MB-468 cells (Figure 11A and 11B). Cells were treated with varying doses of UO126- 2, 5 and 10 μ M for a period of 24 hrs. Treatment with both 5 μ M and 10 μ M dose of UO126 suppressed phosphorylated ERK1/2 levels. Treatment with 10 μ M dose of UO126 also restored radiation sensitivity as assessed by clonogenic cell survival experiments. At this point it was important to see if UO126 can restore radiation sensitivity following short treatments such as 30 minutes. MDA-MB-468 cells were treated with 10 μ M dose of UO126- for a period ranging from 30 minutes to 24 hrs. Treatment with 10 μ M dose of UO126 suppressed phosphorylated ERK1/2 level as early as 30 minutes and also restored radiation sensitivity. For clonogenic cell survival experiments the cells were treated with 10 μ M UO126 for 30 minutes. This treatment was sufficient to restore sensitivity to radiation in the MB468 cells (Figure 12).

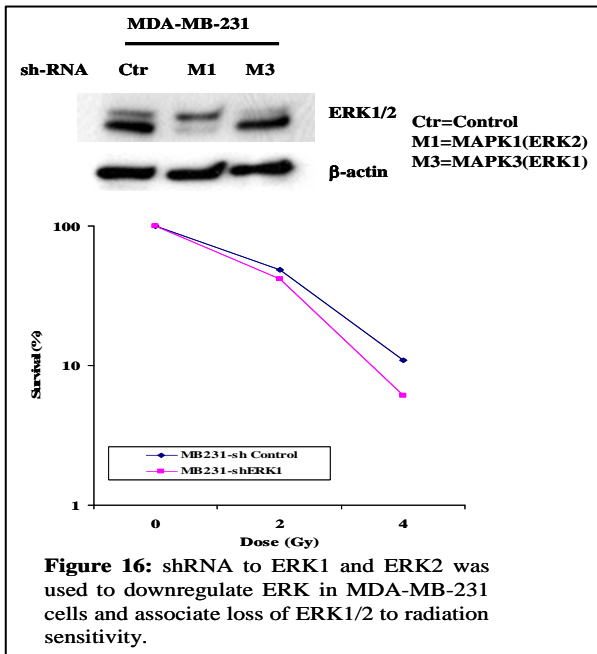
The next question we asked was whether MCF-7 cells which do not constitutively express ERK can be radiosensitized by U0126. To answer this, MCF-7 cells were treated with 10 μ M U0126 for 24 hours and assessed for radiation response by clonogenic cell survival. U0126 treatment had a radioprotective effect on the MCF-7 cells (Figure 13) indicating that the ERK pathway does not mediate the radiation sensitivity of these cells.



survival. U0126 treatment had a radioprotective effect on the MCF-7 cells (Figure 13) indicating that the ERK pathway does not mediate the radiation sensitivity of these cells.



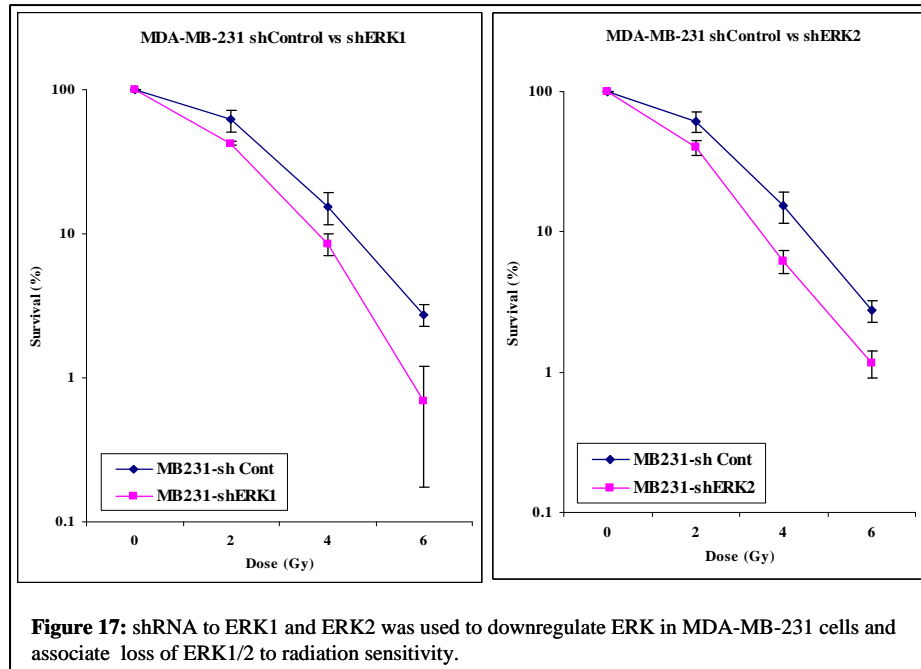
To further test the loss of ER- α with radiation resistance we prepared an MCF-7 cell line in which ER- α levels were knocked down using shRNA to ER- α . MCF-7 cells which are ER- α positive, were stably transfected with shRNA to ER- α and several clones showing downregulation of ER- α were selected and analyzed by western blot analysis (Figure 14). These clones were further expanded and used for examine their sensitivity to radiation. We picked clone #4c for all further experiments as it showed good knockdown of ER- α when compared with the control MCF-7 cells and some of the other clones. Clonogenic cell survival experiments were set up to compare the radiation response of MCF-7 sh-Control cells versus MCF-7 sh-ER- α clone 4c. Knockdown of ER- α made the cells resistant to radiation as can be seen in Figure 15. Additionally, we saw a nice upregulation of ERK in these cells and believe that activated ERK might contribute to radiation resistance in these cells.



As an additional test of our hypothesis we prepared MDA-MB-231 stable clones in which we used a shRNA to knockdown expression of activated ERK1/2. These stable clones were characterized for downregulation of pERK1 or pERK2 by western blot analysis and then tested for their response to radiation. MDA-MB-231 clone with ERK1 knockdown was more sensitive to radiation when compared to the control transfected cells. The degree of

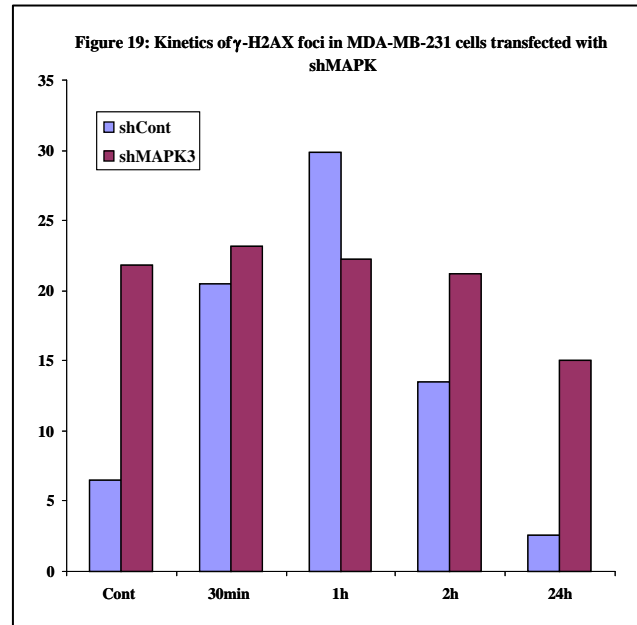
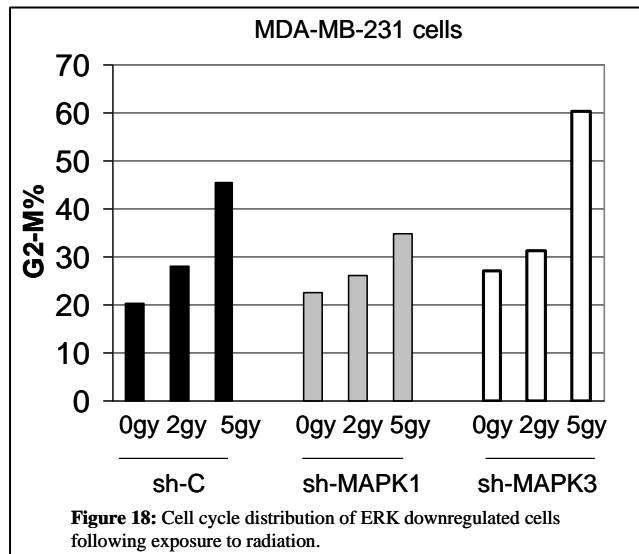
sensitization was less than what we obtained with U0126 but that could be attributed to the fact that U0126 downregulates both ERK1 and ERK2 whereas in the shRNA clone we are knocking down either ERK1 or ERK2 (Figure 16).

Our preliminary IHC done on 3 different cells lines with varying estrogen receptor status



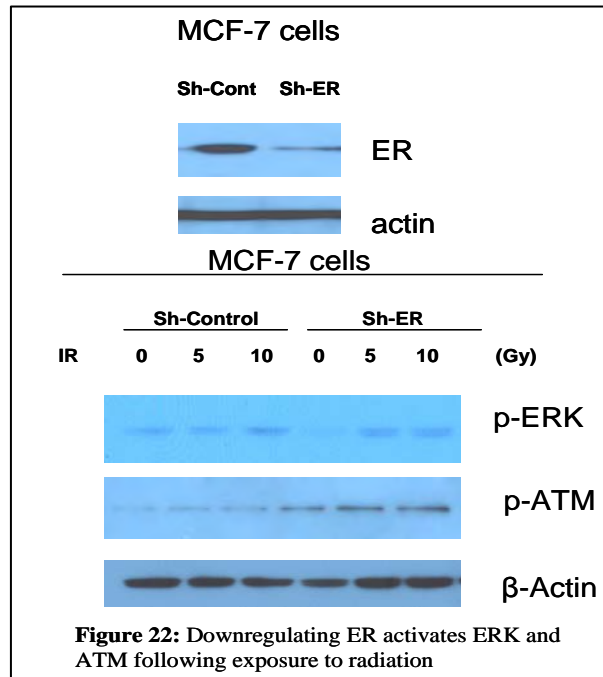
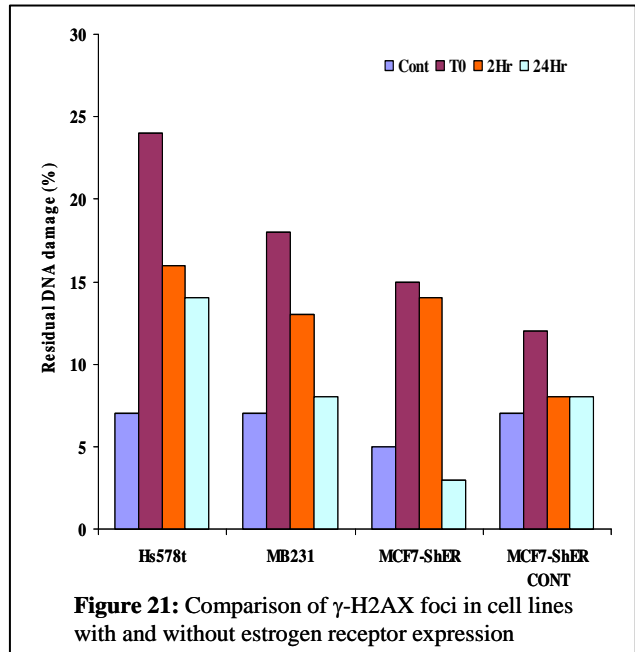
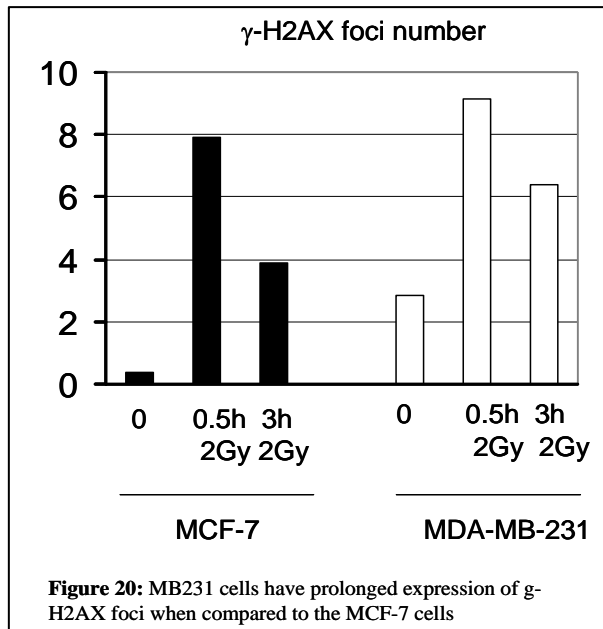
(MDA-MB-231 : ER negative; MCF-7 shER: with estrogen receptor knockdown; and MCF-7 : estrogen receptor positive) demonstrates an abundance of most DNA repair proteins in the ER-negative cells (data not shown). In addition we carried out IHC on commercially available tissue arrays in which the receptor status of the tissue samples is known (data not shown).

We prepared



MDA-MB-231 stable clones in which we used shRNA to knockdown expression of activated ERK1/2. These stable clones were characterized for downregulation of pERK1 or pERK2 by western blot analysis and then tested for their response to radiation. MDA-MB-231 clone with ERK1 knockdown was more sensitive to radiation when compared to the control transfected cells. The degree of sensitization was less than what we have obtained with U0126 but that could be attributed to the fact that U0126 downregulates both ERK1 and ERK2 whereas in the shRNA clone we are knocking down either ERK1 or ERK2. However, we have had to go back and prepare these stable clones again as the insert was lost in the previous cell lines. We analyzed

these clones for differences in radiation sensitivity and the data is shown in Figure 17. Knockdown of both ERK1 and ERK2 radiosensitized MB231 cells to a great extent. We also carried out a cell cycle analysis on these clones and found that knockdown of ERK2 (MAPK1) blocked the cells from entering into G2 phase following 5Gy dose of radiation when compared with sh-control transfected cells. However, shERK1 (MAPK3) enhanced the G2 block compared to the controls (Figure 18).

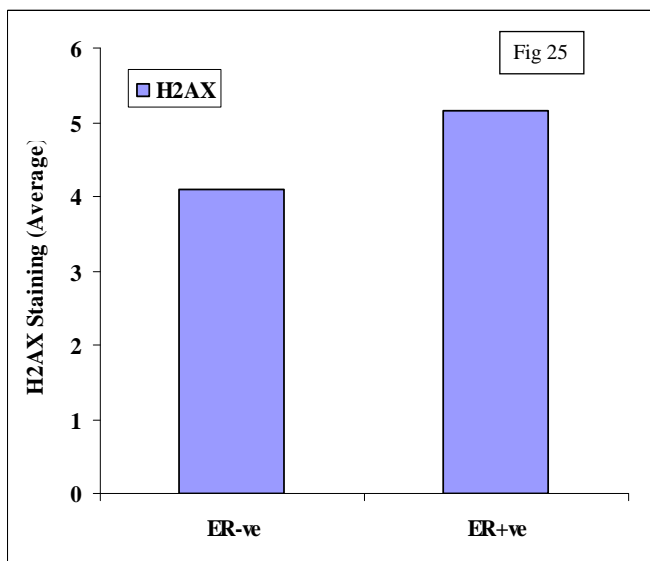
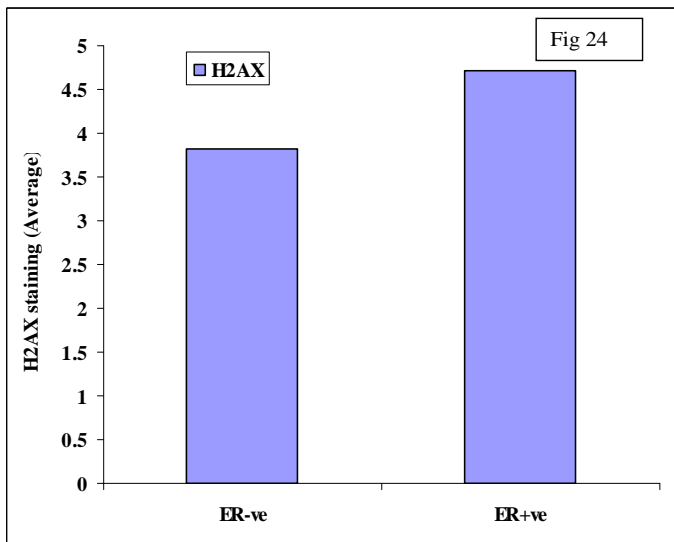
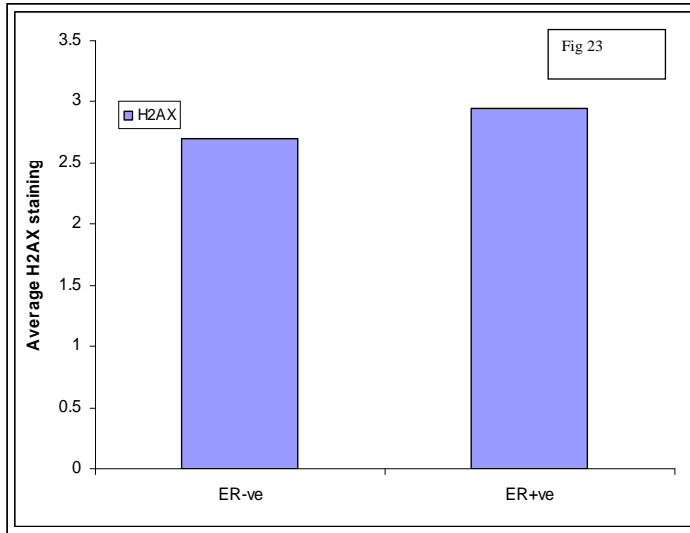


We also analyzed these clones for their DNA repair capacity by studying the kinetics a gamma H2AX foci formation following exposure to 2Gy dose of radiation. As can be seen from figure 19, the shMAPK3 clones had more number of foci to begin with and the foci were prolonged for a longer period of time when compared with the control cells. Similar results were also obtained for the shMAPK1 clones. We also obtained similar results when we compared the ER negative MDA-MB-231 cells with ER-positive MCF-7 cells or MCF-7 in which ER has been downregulated (Figure 20 and 21). MCF-7 cells in which ER expression was knocked down with shRNA also showed an enhanced activation of pATM upon radiation exposure when compared with the shControl cells (Figure 22).

As the major part of this aim was directed towards immunostaining for these DNA repair proteins we spent a lot of time in standardizing our staining protocol. We standardized the staining protocol on 3 different cells lines with varying estrogen receptor status (MDA-MB-231: ER negative; MCF-7 shER: with estrogen receptor knockdown; and MCF-7: estrogen receptor positive). However the staining had to be re-standardized on paraffin embedded cell blocks and on mock tissue arrays. Because of these delays we requested for an extension of the project for a

period of 6 months so that we could complete the staining on the TMA and analyze the data obtained.

Our efforts were directed towards tissue microarray staining for DNA repair markers and



ERK (total and phospho-ERK). Since we were unable to obtain the tissue arrays that we had initially proposed in the grant we obtained commercially available tissue microarrays (TMA) for carrying out the staining. TMAs were obtained from Imgenex. The tumors were infiltrating ductal carcinomas with known estrogen receptor/progesterone receptor status. Tumors were Stage II and III, with normal adjacent tissues available on the array. These TMAs were stained for several different DNA repair proteins including BRCA1, BRCA2, H2AX, Topo-II alpha, and RAD50 and also for the ERK signaling molecule. Staining was carried out in the tissue core lab using an automated stainer and the slides were evaluated by a pathologist in a blinded manner. Intensity of staining was then analyzed using an automated image analysis system and Excel compatible reports were exported. These reports were then analyzed based on the estrogen receptor status of the tissue samples and specific cut-off value of staining for each marker.

Unfortunately, our staining results did not support our initial

hypothesis that ER negative breast cancers have higher levels of DNA repair proteins.

Shown below is the data for γ -H2AX obtained upon staining the TMAs. Including all samples from the TMAs irrespective of the intensity of the staining yielded no significant difference between estrogen receptor negative versus estrogen receptor positive samples. The data is represented as average H2AX staining in the 2 groups (Fig 23). Analyzing the data using a staining intensity of 2 as cut-off (i.e any sample with staining intensity above 2 was considered positive for H2AX) did not change the outcome of the initial results (Fig 24). We further

analyzed the samples by regrouping them on the basis of ER and PR status. Samples which were ER- and PR negative were included in the ER negative group. However any sample that was PR positive was included in the ER-positive group. Upon analyzing these results, again, we found no significant differences in the two groups (results shown below, Fig 25).

We obtained similar results for Topo-II, BRCA1 and BRCA2. We did not observe any differences between the ER negative versus ER positive tumors for any of these markers. One reason for this could be the small number of samples analyzed on the TMAs. However, we plan to submit grant applications in the future in which we will examine the DNA repair proteins in much greater detail and on a larger sample set.

Reportable Outcomes: None

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