High Dose Estrogen Therapy: A Novel Mechanism in Treating Estrogen Receptor Negative Breast Cancer

Parth Patel, Daniel Radin, Donna Leonardi

Abstract— Triple negative breast cancer is a highly aggressive and invasive breast cancer that is difficult to diagnose and treat, since it lacks estrogen, progesterone, and human epidermal growth factor receptors. To treat triple negative breast cancer as well as other forms of breast cancer, researchers are studying high dose estrogen therapy as a potential chemotherapeutic treatment. Clinical trials with high dose estrogen (E2) have shown that it has potential to become a treatment option. However, researchers are hesitant to use it, since its mechanism of action is unknown. The goal of this study was to elucidate the mechanism of action of high dose E2 therapy in reducing proliferation and inducing apoptosis in triple negative breast cancer. In this study, the effect of high dose E2 therapy was studied in relation to PDIA3, a protein localized in the endoplasmic reticulum (ER) whose primary function is to fold proteins. PDIA3 was inhibited by E2 ($p<0.05$), thus preventing the assembly and activation of downstream targets, such as the mammalian target of rapamycin complex 1 (mTORC1) ($p<0.05$), which is involved in inducing cellular proliferation and migration. Thus, a decrease in the activation of mTORC1 suggests the mechanism through which cell proliferation and migration was reduced ($p<0.05$). Furthermore, the inhibition of PDIA3 led to a significant increase in a build up of unfolded proteins ($p<0.05$), which traditionally reside in the lumen of the ER. As a result of increased cellular stress, especially ER stress, the cell was forced into apoptosis, leading to the significant activation of caspase-9 ($p<0.05$), which is a death enzyme involved in apoptosis induced through an intrinsic mechanism. These results provide support for high dose E2 therapy as a potential chemotherapeutic treatment as well as the importance of targeting PDIA3 in triple negative breast cancer cells.

Index Terms— Apoptosis, Caspase-9, Cell Migration, Estrogen, mTORC1, PDI, Triple Negative Breast Cancer, Unfolded Proteins

1 Introduction

TRIPLE negative breast cancer (TNBC) is a highly aggressive and invasive breast cancer that constitutes 15% to 20% of breast cancers [3]. It is unfortunately insensitive to current hormone and targeted antibody treatments due to the lack of estrogen, progesterone, and human epidermal growth factor receptor 2 receptors [18]. Thus, cost-effective therapies cannot be used to treat TNBC [18]. Such molecular and histological distinctiveness of TNBC compel medical professionals to resort to surgery, radiation, or specialized chemotherapies that are generally cost intensive with many adverse side effects [18]. Thus, it is crucial to develop a novel treatment that is effective in treating TNBC.

In recent years, academic researchers as well as those in the pharmaceutical industry are investigating the use of high levels of estrogen (E2) as a potential therapeutic option in TNBC. Interestingly, though E2 is found to promote growth and proliferation, high dose E2 therapy is found to be cytotoxic to breast cancer in some studies [10], [11]. According to Ingle, a synthetic estrogen, known as DES, compared to tamoxifen exhibited no significant difference in terms of response rates as well as time to progression [10]. However, survival rate was significantly higher for women treated with DES [10]. Despite the growing amount of clinical evidence that high dose E2 therapy can be used to treat breast cancer, the mechanism of action of high dose E2 therapy in treating TNBC still remains unclear. As a result, physicians are hesitant to implement this regimen in cancer patients.

Protein disulfide isomerase, family A, member 3 (PDIA3) is part of a group of proteins known as protein disulfide isomerase (PDI) [17]. PDIA3, like other protein disulfide isomerases, is involved in protein folding, specifically in catalyzing the rearrangement disulfide bonds in proteins [17]. Assays using lyophilized proteins have demonstrated that E2 inhibits protein disulfide isomerase [20]. A loss of PDI activity leads to an increase in the production of unfolded proteins in the ER lumen, thus promoting ER stress [8], [9]. High levels of ER stress have been shown to trigger apoptosis [15].

Recent studies suggest that PDIA3 is involved in the assembly of the mammalian target of rapamycin complex 1 (mTORC1) and regulates mTORC1 signaling as well as regulatory-associated protein of mTOR (RAPTOR) expression, most likely due to PDIA3's ability to fold other proteins [17]. Based on the current information regarding PDIA3 as well as E2, it was hypothesized that high dose E2 therapy will inhibit PDIA3. Thus, lower levels of active PDIA3 will be unable to produce sufficient amounts of RAPTOR. Due to low amounts of RAPTOR, mTORC1 will likewise be found in reduced amounts, resulting in less cell proliferation and migration.

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2 Procedure and Methods

2.1 Cell Culture

MDA-MB-231 (ATCC HTB-26), a triple negative breast cancer cell line, and dermal fibroblast (ATCC PCS-201-012), a non-malignant skin cell line, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MDA-MB-231 and dermal fibroblast were cultured in DMEM/F-12 (Invitrogen, Carlsbad, CA). This cell culture media was supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and

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penicillin/streptomycin (Invitrogen), and the cells were incubated at 37ºC with 5% CO_2. Upon reaching 80% confluence, the cells were trypsinized with 0.05% trypsin-EDTA (Invitrogen).

2.2 Exogenous 17 ß -Estradiol Treatment

17 ß-estradiol (E2) (Sigma Aldrich, St. Louis, MO) were prepared in dimethyl sulfoxide (DMSO) (Sigma Aldrich) at 10mM. The stock solution was diluted in DMSO to produce the following concentrations: 1μM, 10μM, 100μM, and 1 mM. The concentration of the 17 ß-estradiol was finally diluted in media in order to obtain the following concentrations: 0.01μM, 0.1μM, 1 μM, and 10μM.

2.3 Cell Viability Assay

Cell Titer 96® Aqueous One Solution Cell Proliferation MTS Assay (Promega, Madison, Wisconsin, USA) was used to determine cell viability of MDA-MB-231 and dermal fibroblast cells after E2 treatment. The cells aforementioned were seeded in a 96-well assay plate at 20,000 cells/well in 100 μL of cell culture medium and were incubated for 24 hours. Then, the cells were treated with E2 concentrations ranging from 0.01 μM to 10 μM for 48 hours. After E2 treatment, 15 μL of Cell Titer 96® Aqueous One Solution was added into each well of the 96-well assay plate, and the 96-well assay plate was incubated at 37ºC, 5% CO_2 for 1 hour. Absorbance was measured at 490 nm using a microplate reader (BioTek ELx808; Winooski, Vermont, USA).

2.4 Preparation of Cell Lysates

The procedure for preparing cell lysates was described previously by Kim [12].

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Primary rabbit polyclonal antibodies for PDIA3 (Cat. No. 15967-1-AP, Proteintech Group, Chicago, IL), RAPTOR (Cat. No. 42- 4000, Invitrogen), and phospho-p70 S6 kinase alpha (Cat. No. sc-11759-R, Santa Cruz Biotechnology, Dallas, TX) were obtained. The ELISA for PDIA3, RAPTOR, and phospho-p70 S6 kinase alpha was performed using the Protein Detector HRP ELISA Kit (KPL, Gaithersburg, MD) as per manufacturer's protocol. The primary antibody and the secondary HRP-linked antibody (KPL) were diluted at 1:300 in 1X BSA (KPL).

2.6 PDI Enzymatic Activity Assay

The modified procedure for conducting the ProteoStat™ PDI assay (Cat. No. ENZ-51024-KP002, Enzo Life Sciences, Farmingdale, NY) with cell lysates was described previously by Anathy [1].

2.7 Detecting Unfolded Proteins

This assay was adapted from Rostagno [18]. After MDA-MB-231 was treated with E2 in a 96-well plate for 48 hours, the cell culture media was removed, and 100 μL of 4% paraformaldehyde was added to each well. The plate was then incubated for 10 minutes at room temperature. Afterwards, the paraformaldehyde was removed, and the wells were rinsed with 100 μL of deionized water. Subsequently, 100 μL of 0.125% solution of Thioflavin S was added to each well. The plate was incubated in the dark for 30 minutes. In order to remove excess fluorochrome, 100 μL of 80% ethanol was added to each well, and the plate was incubated for 10 minutes. Finally, the ethanol was removed, and 100 μL of PBS was added to each well. The fluorescence was read on a Synergy HT microplate reader (BioTek, Winooski, VT) with excitation/emission at 495/519nm.

2.8 Caspase-9 Assay

Following previously described E2 treatment in 96 well plates, 100 μL of Caspase- Glo Reagent (Promega) for Caspase-9 were added to the appropriate wells. The plate was incubated at 37°C, 5% CO_2 for three hours. The luminescence was read on a Synergy HT microplate reader (BioTek).

2.9 Migration Assay

The effect of E2 on MDA-MB-231 cell migration was measured using a 96-well chemotaxis chamber assay (Chamber Series Neuroprobe, Cabin John, MD). In a 6-well plate, MDA-MB-231 was seeded at 590,000 cells/well in 2 mL of cell culture medium and was incubated for 24 hours. Then, the cells were treated with E2 concentrations ranging from 0.01 μM to 10 μM for 48 hours. After 48 hours of treatment, cells were trypsinized with 0.05% trypsin-EDTA. In the chamber, 10,000 cells/well in 100μL of serum-free cell culture medium were plated in the upper wells. In the lower wells, the chemoattractant, cell culture medium with serum-rich, was plated at 400μL/well. The chamber was incubated at 37ºC, 5% CO_2 for 24 hours. In this assay, cell migration was defined as the number of cells that migrated through 8-mm-pore polyvinyl pyrrolidone-free polycarbonate filter (Neuroprobe) coated with collagen I (40 mg/ml; Becton Dickinson, Bedford, MA) after 24 hours of incubation. After the 24-hour incubation period, the filter was removed, fixed with 95% methanol, and stained with Giemsa stain (Sigma-Aldrich). Nonmigrated cells on the opposite side were removed with a cotton swab. Afterwards, parts of the filter were mounted on a glass slide. Five replicates were created for each concentration, and ten random fields of visions were counted at 100x magnification. Part of this procedure is adapted from Zhou [23].

2.10 Data Analysis

Data from the assays were analyzed using Microsoft Excel: MAC 2011 (Microsoft, Redmond, Washington, USA) and StatPlus: Mac (AnalySoft). For all assays, the number of replicates (n) were greater than or equal to three with a sample size of each replicate being greater than or equal to five. The unpaired Student's t-test (t-test) was used to determine statistical significance, in which the alpha value was set at 0.05. One-way analysis of variance (ANOVA) was conducted to determine the presence of a dose-response, in which the alpha value was also set at 0.05. One-way analysis of covariance (ANCOVA) was conducted to determine statistical significance between MDA-MB-231 and dermal fibroblast, in which the alpha value was set at 0.05.

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3 RESULTS

Effect of E2 on cell viability

Estrogen therapy on dermal fibroblasts yields a biphasic dose-response. High dose E2 (10uM) only inhibits cell proliferation by 6% ($p>0.05$), suggesting this form of treatment is not significantly cytotoxic to healthy tissue at this dose. Interestingly, exogenous E2 treatment produces a dose-dependent decline in cell viability in MDA-MB-231 cells (ANOVA, $p<0.05$). 10 uM E2 inhibits cell proliferation by 24% ($p<0.01$), a four-fold increase in growth inhibition as compared to dermal fibroblast cell viability following E2 treatment. When compared to dermal fibroblast, MDA-MB-231 has a significantly lower cell viability (ANCOVA, $p<0.005$).

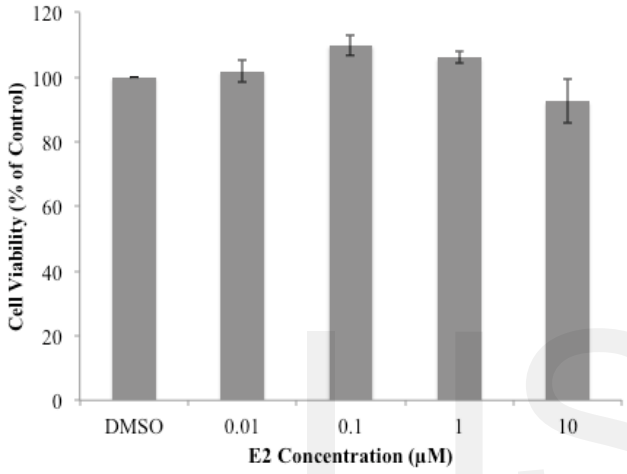

Fig 1. Effect of E2 on dermal fibroblast cell viability. Dermal fibroblasts exhibit no significant decline in cell viability at the highest dose. Bars are means ± SE (n=3).

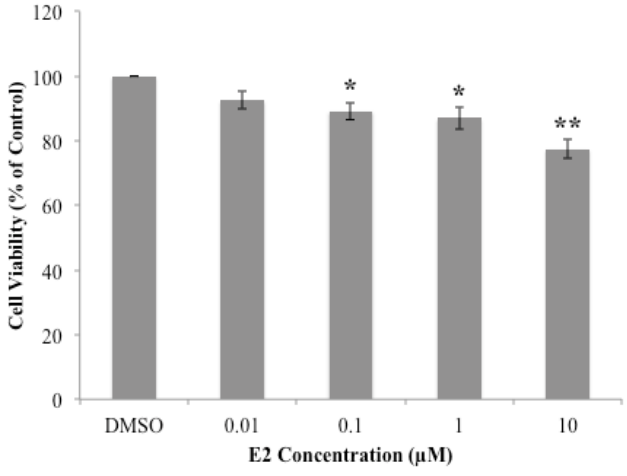

Fig 2. Effect of E2 on MDA-MB-231 cell viability. E2 inhibits cell viability in a dose-dependent fashion. Bars are means ± SE (n=3). ANOVA, $p<0.05$. *$p<0.05$, **$p<0.01$, t-test, comparing data to DMSO control.

Effect of E2 on global PDI activity

In accordance with our hypothesis, high dose E2 inhibits global PDI activity in a dose-dependent decline in MDA-MB-231 (ANOVA, $p<0.05$). These results confirm the mechanism of action of E2 in estrogen receptor negative breast cancers. The highest dose of E2 significantly inhibits PDI activity by approximately 20% ($p<0.005$).

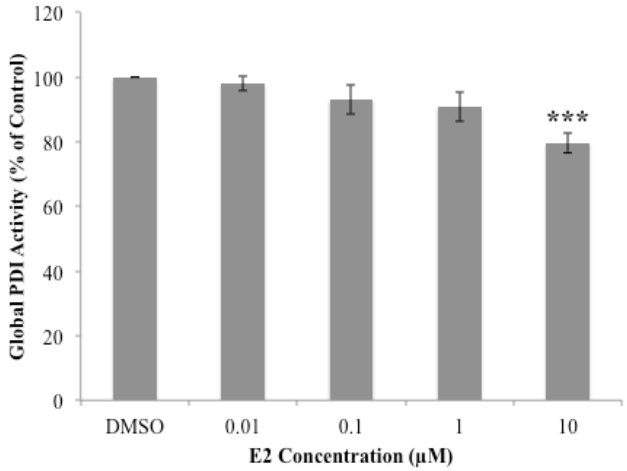

Fig 3. Effect of E2 on global PDI activity. High dose E2 significantly inhibits PDI activity in MDA-MB-231. Bars are means ± SE (n=4). ANOVA, $p<0.05$. ***$p<0.005$, t-test, comparing data to DMSO control.

Effect of E2 on PDIA3 levels

While previous studies have investigated the effects of E2 on global PDI levels, it was interesting to see how PDIA3 levels were affected in the presence of E2. After 48 hours of E2 treatment, a significant decrease in PDIA3 levels was seen in 0.1 µM, 1 µM, and 10 µM. At the highest concentration of E2, there was a 15% decrease in PDIA3 levels, ($p<0.01$). Due to such a decrease in protein levels and activity, E2 has a drastic effect on the downstream products of PDIA3. In MDA-MB-231, expression may not have changed, but the concentration of properly folded PDIA3 may have decreased due to a feedback loop, which began by its inhibition.

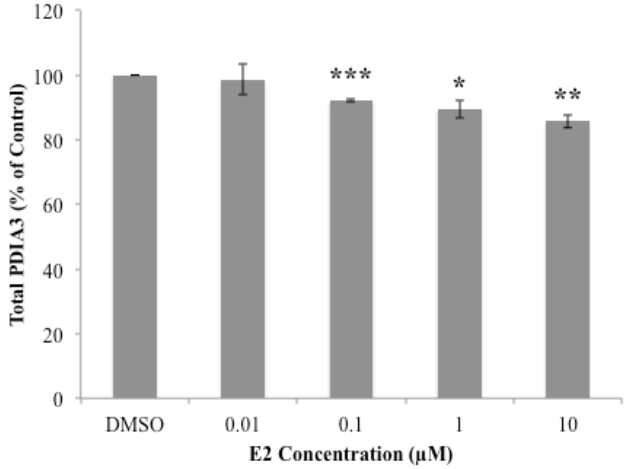

Fig 4. Effect of E2 on PDIA3 levels. High dose E2 significantly reduces PDIA3 levels for most of the doses. Bars are means ± SE (n=3). *$p<0.05$, **$p<0.01$, ***$p<0.005$, t-test, comparing data to DMSO control.

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Effect of E2 on RAPTOR production and mTORC1 activity

After it was determined that E2 treatment attenuates PDI activity, it was interesting to determine what effect this attenuation had on RAPTOR production and overall mTORC1 activity. PDIA3 is responsible for properly folding RAPTOR into its functional form, and RAPTOR is an integral component in mTORC1. Results indicate that E2 treatment inhibits RAPTOR production in a dose-dependent fashion (ANOVA, $p<0.005$) while also inhibiting mTORC1 activity at the highest dose ($p<0.05$).

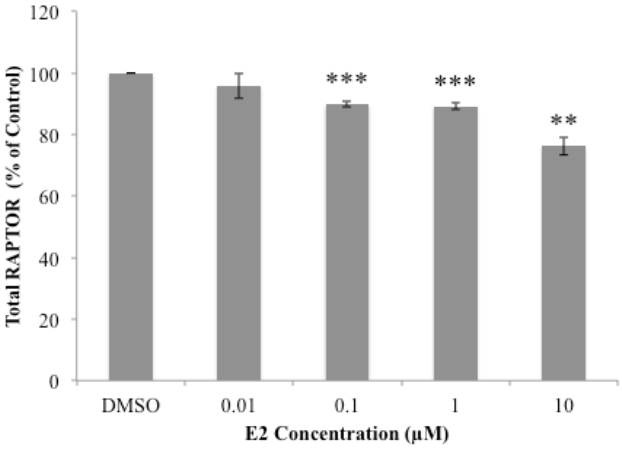

Fig 5. Effect of E2 on total RAPTOR. E2 inhibits RAPTOR production in a dose-dependent fashion. Bars are means ± SE (n=3). ANOVA, $p<0.005$. *$p<0.05$, **$p<0.01$, ***$p<0.005$, t-test, comparing data to DMSO control.

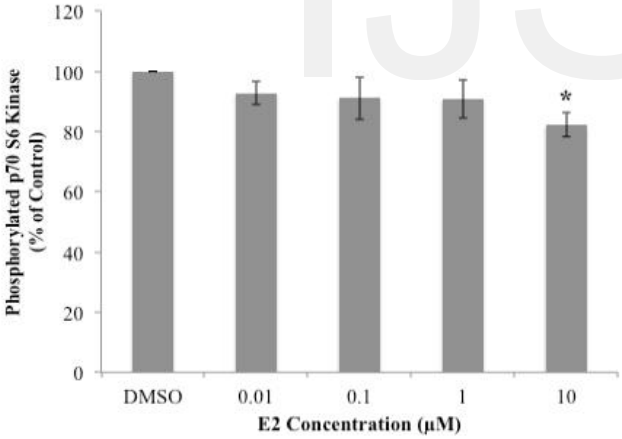

Fig 6. Effect of E2 on mTORC1 activity. mTORC1 activity was measured by determining how much p70 S6 kinase was phosphorylated, which is a downstream product of mTORC1. E2 inhibits p70 S6 kinase phosphorylation at the highest dose. Bars are means ± SE (n=3). *$p<0.05$, t-test, comparing data to DMSO control.

Effect of E2 on caspase-9 activity

Although the apoptotic mechanism of E2 has not been heavily investigated, E2 administration triggers apoptosis in MDA-MB-131 in a dose-response fashion ($p<0.005$). Furthermore, since caspase-9 significantly increased in activity at 10 µM, it is clear that the cause of apoptosis was intrinsic ($p<0.01$).

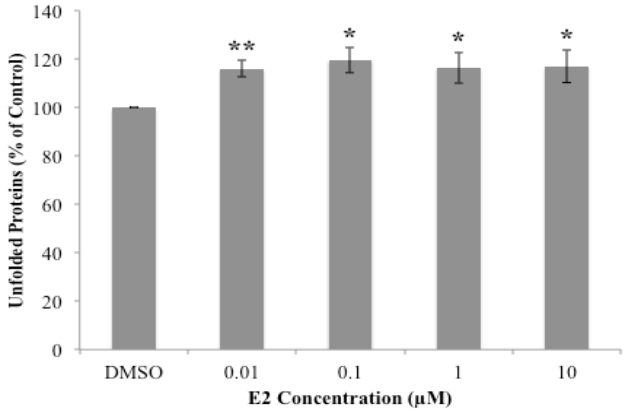

Fig 7. Effect of E2 on caspase-9 activity. E2 treatment increases caspase-9 activity in a dose-dependent fashion. Bars are means ± SE (n=3). ANOVA, $p<0.005$. *$p<0.05$, **$p<0.01$, t-test, comparing data to DMSO control.

Effect of E2 on unfolded protein production

Following E2 treatment, the amount of unfolded proteins per E2 concentration was measured. From the lowest concentration to the highest, there is a significant increase in unfolded proteins ($p<0.05$). This assay also confirms that with lowered amounts of PDI activity, the cell experiences a buildup of improperly folded proteins. According to Beriault, detection of unfolded proteins through such thioflavin dyes are indicative of ER stress [2].

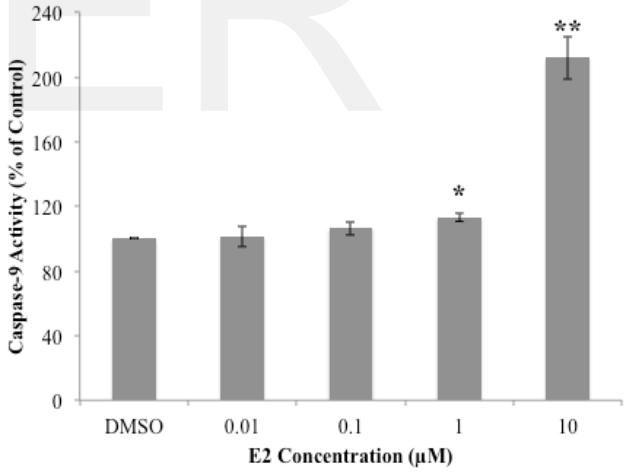

Fig 8. Effect of E2 on unfolded protein production. E2 treatment increases unfolded protein production. Bars are means ± SE (n=4). *$p<0.05$. **$p<0.01$, t-test, comparing data to DMSO control.

Effect of E2 on cell migration

After determining that exogenous E2 treatment diminished mTORC1 activity, it was interesting to determine the effect it had on cell migration to serum-rich media, as mTORC1 has been implicated in a cell's ability to migrate [14]. In accordance with diminished mTORC1 activity, high-dose estrogen reduced this cancer's ability to migrate by 42% ($p<0.05$). Previous reports have never established this novel relationship between E2 and migration to serum-rich media.

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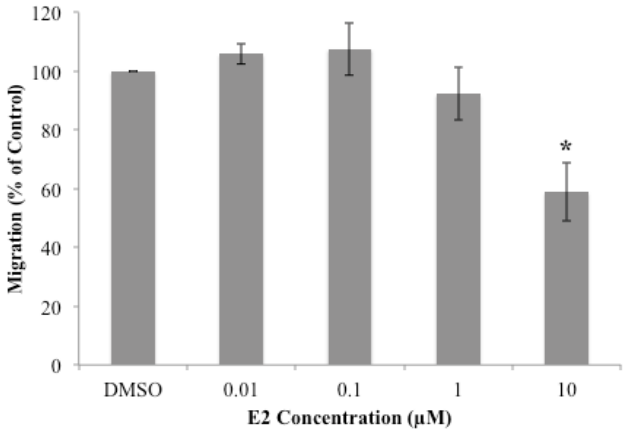

Fig 9. Effect of E2 on cell migration. Migration of MDA-MB-231 cells from serum-free to serum-rich media was measured following E2 treatment. E2 inhibits migration at the highest dose. Bars are means ± SE (n=4). ANOVA, $p<0.005$. *$p<0.05$, t-test, comparing data to DMSO control.

4 Discussion

While hormone, targeted antibody, and certain chemotherapeutic treatments can be used to treat estrogen-receptor positive breast cancer, these treatments options are ineffective in patients with TNBC [18]. As a result, it is crucial to develop alternative medication that have high efficacy in TNBC. Thus, researchers are investigating high dose E2 therapy as a potential chemotherapy in patients with TNBC. Clinical studies have proven that high dose E2 can be a potential chemotherapeutic drug [10]. In certain studies, high dose E2 therapy has been shown to have greater efficacy as compared to certain leading chemotherapy [10]. Despite such studies, high dose E2 therapy is not implemented in patients with TNBC, as the mechanism of action is not fully understood.

Prior to understanding the effects of E2 on TNBC, it was crucial to examine the effect of E2 on non-malignant cells, since chemotherapeutic compounds often come in contact with them. At lower doses of E2, cellular proliferation remains constant or is partially promoted. At 10 µM E2, the decrease in cell viability is not significant, suggesting that the highest dose of E2 does not have a substantial effect on dermal fibroblast (Figure 1). As for MDA-MB-231 (Figure 2), cell viability decreases in a dose response fashion ($p<0.01$). At 10 µM of E2, there was 23% decrease in cell viability and was significantly lower when compared to the solvent control ($p<0.01$). When compared to dermal fibroblast, MDA-MB-231 has significantly lower cell viability ($p<0.005$). The variation in cell viability between MDA-MB-231 and dermal fibroblast can possibly be attributed to the physiology of these two cells types. However, the role of PDIA3 in MDA-MB-231 and dermal fibroblast need to be further studied to determine to what extent this proposed mechanism is true.

While measuring cell viability revealed the overall effect of E2 on MDA-MB-231, it was critical to determine whether E2 worked through the proposed mechanism. In this study, PDIA3 was identified as a potential target for E2, through which E2 is able to elicit a novel anti-proliferative response. Since PDIA3 activity directly correlates with global PDI activity, global PDI activity was measured in the presence of high dose E2. At 10 µM E2, there is a significant decrease in global PDI activity, with only 80% active PDI ($p<0.005$). Such a decrease in PDI activity reconfirms the study conducted by Tsibris that E2 acts as a functional inhibitor, selectively inhibiting the isomerase activity of PDI [20]. While previous studies have investigated the effects of E2 on global PDI levels, it was interesting to see how PDIA3 levels was affected in the presence of E2. As seen in Figure 4, at 10 µM E2, PDIA3 decreases by approximately 13% ($p<0.05$). Such a decrease in PDIA3 levels is most likely not due to a change in expression. Instead, the concentration of properly folded PDIA3 may have decreased due to a feedback loop, which began by its inhibition. However, this requires further study of PDIA3 expression in order to understand such a decrease in the quantity of PDIA3.

After investigating the effects of E2 on PDI, it was critical to see whether there were any changes in mTORC1 levels. After 48 hour of E2 treatment, total RAPTOR levels decreased in a dose-response fashion ($p<0.05$). Interestingly, at 10 µM E2, there was approximately 25% decrease in RAPTOR levels ($p<0.01$). Such findings were in accordance with the study conducted by Ramirez-Rangel, in which a decrease in active PDIA3 led to a reduction of RAPTOR [17]. As hypothesized, a decrease in RAPTOR levels led to a decrease in active mTORC1. As seen by Figure 6, phosphorylated p70 S6 kinase levels significantly decreased ($p<0.05$). A decrease in phosphorylated p70 S6 kinase suggests that there is a decrease in active mTORC1 levels, since p70 S6 kinase is a downstream product of mTORC1. As a result, cell proliferation is inhibited in MDA-MB-231 in the presence of 10 µM E2.

While the RAPTOR and phosphorylated p70 S6 kinase levels provided an understanding regarding cell proliferation, it was crucial to properly comprehend the reason for cell death. It was vital to understand whether cell death occurred due to apoptosis or necrosis. In order to properly assess such characteristics of E2, activated caspase-9-a death enzyme that induces an internal signal for apoptosis - was measured in the presence of E2. As seen in Figure 7, with increasing doses of E2, caspase-9 levels increase in a dose response fashion ($p<0.001$). At 10 µM E2, there is a 124% increase in caspase-9 levels ($p<0.01$). Such results suggest that at the highest concentration, apoptosis is induced, and the signal for apoptosis is intrinsic. While measuring caspase-9 levels provided an understanding of apoptosis, this measurement solely confirmed the downstream effects of exogenous E2 treatment. It was hypothesized that apoptosis will be promoted in the presence of E2 due to an increase in unfolded proteins. Since PDI, including PDIA3, is primarily involved in folding other proteins, its inhibition by E2 will increase the amount of unfolded proteins, subsequently promoting greater stress in the endoplasmic reticulum (ER). Based on this hypothesis, levels of unfolded proteins were measured in the presence of E2. Since total unfolded proteins cannot be quantified, relative levels of aggregates of beta-pleated sheets were measured using Thioflavin S. In the presence of all E2 concentrations, the levels of unfolded proteins are significantly higher ($p<0.05$). Such results suggest

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that from the lowest E2 concentration to the highest, unfolded proteins significantly increase, most likely due to the decrease in PDI activity. As reported by Beriault, an increase in unfolded proteins detected by such thioflavin-based dyes signify an increase in ER stress [2]. At high levels, ER stress has been known to induce apoptosis [2].

After studying the relationship between PDIA3 and mTORC1, it was interesting to see whether high dose E2 had any effect on the migratory abilities of MDA-MB-231 since mTORC1 activity modulates growth factor induced cell migration [14]. As a result, perturbation of this complex should inhibit migration to serum rich media. As seen in Figure 9, the migration of MDA-MB-231 toward nutrient rich media decreased in a dose response fashion in the presence of E2 ($p<0.005$). Interestingly, at the highest E2 concentration, migration decreased by approximately 42% ($p<0.05$). Such results suggest the widespread advantages of high dose E2. High dose E2 does not only significantly reduce cell viability but decreases the ability of MDA-MB-231 to migrate toward a chemoattractant. Figure 10 depicts the possible mechanism of action of high dose E2 elucidated by this study, which can be another approach to treating TNBC. Despite such possibilities, the effect of E2 in *in vivo* must be further studied to determine to what extent this holds true.

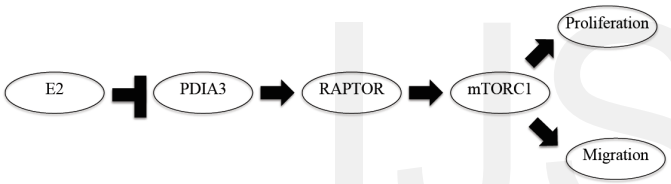

Fig 10. Schematic pathway of E2's possible mechanism of action in MDA-MB-231 (estrogen receptor negative) cells.

This study provided a greater understanding on the various interactions PDI, especially PDIA3, share with other proteins and complexes, such as mTORC1. Despite the various novel findings that emerged from this study, there are questions regarding PDIA3 that still need to be answered. For future research, it would be interesting to further understand the level of endoplasmic reticulum stress that exist at high doses of E2. While an increase in unfolded proteins was seen in the presence of E2, it would be interesting to see to what extent the ER is promoting apoptosis. The role of the ER in promoting apoptosis can be investigated through the unfolded protein response (UPR). The unfolded protein response is activated in the presence of high levels of unfolded proteins in the ER. This response is responsible for either trying to restore ER homeostasis or inducing apoptosis if the stress is too high. By investigating this pathway, a deeper understanding of whether the UPR or another signal transduction pathway plays a role in inducing apoptosis. Furthermore, the aforementioned proposed pathway should be studied in greater detail in non-malignant tissue to further understand the differences in cell viability between MDA-MB-231 and non-cancerous cells after 48 hour of E2 treatment. While proteins, such as PDIA3, mTORC1, and RAPTOR are present in MDA-MB-231 as well as dermal fibroblast, both cells behave quite differently in the presence of E2. This can possibly be due to the way both cells interact with E2. However, in order to adequately evaluate this hypothesis, a comparison of the expression, mRNA level, and protein activity of PDIA3, RAPTOR, and mTORC1 is needed in MDA-MB-231 and dermal fibroblast.

5 Conclusion

In recent years, numerous clinical studies have shown the efficacy of high dose E2 in treating breast cancer [10]. Despite the growing amount of clinical evidence, the medical community is hesitant to treat patients with high dose E2, since the mechanism of action is not fully understood. This study not only investigated the efficacy of high dose E2 in breast cancer but also a novel mechanism of action of E2 in treating TNBC. The efficacy of high dose E2 is suggested to be due to its inhibition of PDIA3. The inhibition of PDIA3 led to a subsequent decrease in RAPTOR levels as well as mTORC1 activity, thus limiting the cellular proliferation and migration of TNBC cells. Additionally, the inhibition of PDIA3 increased unfolded proteins, most likely also increasing ER stress. At a high E2 dose, TNBC cells were not able to reduce this stress, forcing them to induce apoptosis via caspase-9. While anti-proliferative effects were seen in TNBC cells, dermal fibroblasts were not detrimentally affected by E2. In all, this study provides support for high dose E2 as a potential chemotherapeutic drug in treating TNBC.

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