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P53 mediates estradiol induced activation of apoptosis and DNA repair in non-malignant colonocytes

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ABSTRACT

Clinical and animal studies have shown a strong link between estrogen status in women and decreased risk of colon cancer. However, little research has been done into the mechanism of protection that estrogen provides. Our laboratory has demonstrated that estradiol (E_2) inhibits the development of pre-neoplastic lesions through an estrogen receptor β (ERβ) mediated mechanism in mice. Our data also suggest that the primary protective role of E_2 treatment is increased apoptosis in non-malignant colonocytes that are damaged and at risk of becoming cancerous. The p53 protein plays a crucial role in the cellular response to stress by inducing cell cycle arrest, DNA repair mechanisms, and/or apoptosis. Due to the observed induction of apoptosis in response to E_2, we are investigating the role of p53 in this chemoprotective mechanism. E_2 suppressed growth of young adult mouse colonocytes (YAMCs) by inducing apoptosis and these physiological responses were completely lost in YAMCs lacking a functional p53 protein. Western blot analysis demonstrated increases in p53 protein levels in YAMCs after treatment with E_2 likely due to protein stabilization. E_2 was shown to enhance the transcriptional activity of p53, resulting in up-regulation of pro-apoptotic p53 target genes (Bax, Noxa, and PUMA). Finally, repair of DNA double stranded breaks was shown to be increased by E_2 treatment. Collectively, these data are the first to demonstrate that p53 is a primary mediator of the protective actions of E_2 in the colon.

1. Introduction

In general, women have a reduced risk of developing colon cancer [1]. Data from The Women's Health Initiative study suggests that post-menopausal women receiving hormone replacement therapy (HRT) have a lower incidence of the disease compared to the placebo group [2]. Several other clinical studies have generated data supporting both HRT and estrogen replacement therapy (ERT) as viable interventions for the reduction of colon cancer risk in post-menopausal women [3–6]. Animal studies offer further evidence supporting the theory that estrogens may play a protective role against the development of colon cancer. Estradiol (E_2) treatment in ovariectomized (OVX) rats suppressed tumor development in a dimethylhydrazine-induced model of colon cancer [7], and orally administered estrone reduced colon tumor number in azoxymethane (AOM) treated mice [8]. Additionally, our laboratory has demonstrated E_2 treatment in mice reduced the formation of pre-neoplastic lesions [9], suggesting that estrogenic protection occurs at an early stage of tumor development. This effect is lost in estrogen receptor β (ERβ) knockout mice indicating this is likely a receptor mediated event. Our previously reported data point towards increased apoptosis within the colonic crypts of animals exposed to carcinogen as a major mechanism of E_2 mediated protection against colon carcinogenesis [9].

While *in vivo* studies have produced promising data supporting estrogen as a chemoprotective agent against the development of colon cancer, the majority of *in vitro* studies have not shown similar results [10–14]. This is likely due to the use of colon cancer cell lines as the most common models for *in vitro* experiments. As a result, our laboratory has begun to characterize how non-malignant colonocyte cellular physiology is altered by E_2. The Young Adult Mouse Colon (YAMC) cell line has been successfully used as an *in vitro* model to investigate the physiological responses of non-malignant colonocytes to stimuli, such as poly-unsaturated fatty acids and carbon monoxide [15,16]. Our data have shown that YAMCs do in fact respond to E_2 treatment in a manner that is predictive of what occurs in non-malignant colonocytes *in vivo* [9]. Furthermore, the ability of these cells to exhibit physiological changes due to E_2 treatment is lost with malignant transformation. Together, these data suggest a chemo-protective role for E_2 in the non-malignant colon, and warrant further study into the estrogenic mechanism of action in colonocytes.

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The process of cancer progression often involves loss of heterozygosity of wild type *p53* or dominant negative mutations in this gene. Point mutations in *p53* occur in approximately 50% of colorectal carcinomas, and these mutations are often associated with disease severity [17–21]. The p53 protein is a tumor suppressor and triggers a wide array of physiological functions within the cell in response to DNA damage, aberrant growth signals, and oxidative stress [22]. These include initiation of cellular senescence, induction of apoptosis, and initiation of DNA repair mechanisms in response to varying forms of cellular stress, such as DNA damage [23]. E_2 treatment has been shown to alter p53 protein levels and expression of its downstream targets when ERβ is present in MCF-7 breast cancer cells, HC11 murine breast epithelial cells, HHUA endometrial carcinoma cells and osteoblasts [24–27]. The relationships between E_2 treatment and p53 activity seen in these and other cell types, along with previously observed apoptosis in YAMCs, suggest that E_2 triggered p53 activity can play an important role in the cellular response to DNA damage. As such, the E_2 induced up-regulation of p53 activity is likely to have an important function in the normal biological activity of non-malignant colonocytes *in vitro*, and the data reported herein begin to elucidate the role p53 plays in estrogenic chemo-protection within the colon.

2. Materials and methods

2.1. Cells

YAMC bleo/neo and mp53/neo cells were provided by Dr. Hartmut Land (University of Rochester Medical Center). For general maintenance, cells were cultured in RPMI 1640 (Sigma Aldrich) with 10% fetal bovine serum (FBS; HyClone); 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences); and 1% gentamicin (GIBCO). Cells were maintained under permissive conditions, 33 °C with 5 units γ-interferon (IFNγ)/mL medium (Roche) on plates coated with rat tail collagen type I (BD Biosciences). Forty-eight hours before plating for all experiments, YAMC bleo/neo and mp53/neo cells were transferred to medium containing 10% charcoal-dextran stripped FBS, 1% gentamicin, and 0.1% ITS. β-Estradiol and Fulvestrant, ICI 182,780, (ICI) (Sigma Aldrich) were diluted in dimethyl sulfoxide (DMSO) as 1000× stocks and delivered as 1 μL/mL medium to achieve the final dose listed.

2.2. Cell number assay

YAMC bleo/neo and mp53/neo cells were seeded at 15,000 cells per well on six-well plates and grown under non-permissive conditions, 37 °C and absence of IFNγ. Cells were exposed to individual treatments of vehicle or 1 nM E_2 alone or in combination with 1 μM ICI for 96 h, and 48 h after the first treatment, the medium was changed and a second dose of the given treatments was delivered. At the end of the 96-h treatment period, cells were trypsinized and prepared for counting. Cell concentration was determined using a Beckman Coulter particle counter. Twenty micro-liters of sample were diluted in 10 mL Isotone II diluent (Beckman Coulter), and each sample was counted thrice. Three wells per treatment per experiment were used and three replicate experiments were conducted.

2.3. Apoptosis

YAMC bleo/neo and mp53/neo cells (15,000/well) were seeded on six-well plates and grown in stripped serum medium under non-permissive conditions. Cells were treated for 96 h, with vehicle or 1 nM E_2 treatments changed every 48 h. At the end of the treatment period, cells were trypsinized and collected. After collection, cells were centrifuged and the medium was replaced with lysis buffer from the EnzChek Caspase-3 Assay kit no. 2 (Invitrogen). The Invitrogen protocol was followed for this procedure. Apoptosis was measured as increased fluorescence measured on a TECAN infinite M200 plate reader. Three wells per treatment per experiment were used and four replicate experiments were conducted.

2.4. p53 transcriptional reporter assay

The PG13-Luc transcriptional reporter contains 13 copies of the p53 response element (5′-CCAGGCAAGTCCAGGCAGG-3′) driving expression of the luciferase gene [28]. YAMC bleo/neo cells (15,000/well) were seeded in 12-well plates and maintained at 33 °C in stripped serum medium. Cells were grown for 72 h under non-permissive conditions. After 48 h, cells were transiently transfected with plasmids containing β-galactosidase and the pG13 p53-luciferase reporter construct. Transfection was performed using the Effectene transfection reagent (Qiagen) using 0.5 μg β-gal and 4 μg pG13 per plate. Cells were treated with 1 nM E_2 or vehicle for the final 0, 4, 8, or 24 h vehicle or 1 nM E_2 alone or in combination with 1 μM ICI for the final 18 h. Media was then removed from cells, and the cells washed with PBS and lysed using 200 μL Reporter Lysis Buffer (Promega). Cell lysates were combined with Luciferase reagent or β-galactosidase reagent (Promega) and activity measured on a TECAN infinite M200 plate reader.

2.5. Protein measurements

YAMC bleo/neo cells (25,000/well) were seeded in 6-well plates and maintained at 33 °C in stripped serum medium. Cells were grown for 72 h under non-permissive conditions and treated with 1 nM E_2 or vehicle for the final 0, 4, 8, or 24 h. Protein was extracted by adding 100 μL lysis buffer to the flask for 30 min at room temperature. After incubation, solution was mixed gently with a pipette and the contents were transferred to microcentrifuge tubes. Total protein was quantified by UV spectrometry. Protein concentration was determined by Western blot using the Immobilon Western Chemiluminescent Horseradish Peroxidase (HRP) Substrate kit (Millipore); the methodology was previously described [29]. Antibodies used were p53 polyclonal antibody (905-510), Mdm2 polyclonal antibody (905-462), goat anti-rabbit IgG (Assay Designs), and ERβ antibody (ab3577, Abcam).

2.6. RT-PCR.

YAMC bleo/neo and mp53/neo cells (25,000/well) were seeded in 6-well plates and maintained under non-permissive conditions in stripped serum media. Cells were grown for 72 h and treated with 1 nM E_2 or vehicle for the final 24 h. Cells were trypsinized and centrifuged, and RNA isolation conducted using the RNAqueous-4PCR kit (Ambion). 1 μg total RNA was used for cDNA synthesis using the Transcriptor First Strand cDNA Synthesis kit (Roche). RT-PCR samples contained 9.5 μL FastStart Universal SYBR Green Master Mix (Roche), 1.25 μL forward and reverse primers (18s F-TCA AGA ACG AAA GTC GGA GGT T, 18s R-GGA CAT CTA AGG GCA TCA CAG, p53 F-AAA GAA AAA ACC ACT TGA TGG AGA GT, p53 R-CGG AAC ATC TCG AAG CGT TTA, Mdm2 F-TGA ATC CTC CCC TTC CAT CA, Mdm2 R-TCG TCT GGA AGC CAG TTC TCA, Bax F-CAC CAG CTC TGA GCA GAT G, Bax R-GCG AGG CGG TGA GCA CTC C, Bcl-2 F-ATC TTC TCC TTC CAG CCT GA, Bcl-2 R-TCA GTC ATC CAC AGG GCG AT, NOXA F-GAA ATG CCT GGT ATT GGA TGG A, NOXA R-GAA CTC AT CCT ATC TCC TTC ATC AT, p21 F-TTC CGC ACA GGA GCA AAG T, p21 R-CGG CGC AAC TGC TCA CT, p27 F-GGC CAA CAG AAC AGA AGA AAA TGT, p27 R-GGG CGT CTG CTC CAC AGT or PUMA F-GCG GCG GAG ACA AGA AGA, PUMA R-GGA GTC CCA TGA AGA GAT TGT ACA;

Sigma–Aldrich), 11 μL RNase-free water, and 2 μL cDNA. RT-PCR was run on a Bio-Rad iQ5 thermocycler for 45 cycles.

2.7. DNA damage assay

YAMC bleo/neo and mp53/neo cells (15,000/well) were seeded on 22 mm × 22 mm cover slips in six-well plates and grown in stripped serum medium under non-permissive conditions. Cells were exposed to vehicle or 1 nM E_2 alone or in combination with 1 μmol/L ICI for 72 h. At the end of the treatment period, cells were irradiated with 2 Gy followed by a 1 h incubation period at 37 °C. Cells on cover slips were then fixed in 4% paraformaldehyde for 10 min. Cells were then incubated overnight at 4 °C with phosphohistone H2A.X primary antibody (Cell Signaling). Alexa Fluor 488 goat anti-rabbit IgG and Prolong Gold + DAPI (Invitrogen) were used as secondary antibody and nuclear counterstain, respectively. Visualization of the stain was conducted using a Zeiss Axiovert 200 microscope with Axiocam MRc. The number of foci/cell was counted in 30 cells per slide.

2.8. Statistical analysis

All data are presented as mean ± SEM. Experiments were conducted in triplicate and repeated three times. Data for cell growth experiments were transformed to percentage of control to eliminate false error that might be introduced based on differences in average cell number between replicates. Analysis for all data was determined using a student *t*-test. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Wild type p53 protein is required for E_2 regulation of cell number

The YAMC cell line was originally derived in the Whitehead laboratory from normal colonic epithelial cells isolated from the *H-2K*^{b}-tsA58 transgenic mouse [30]. These cells are conditionally immortalized by SV40 inactivation of p53, but while under non-permissive conditions (described in Section 2.1) p53 concentrations and its activity return to normal. Our previous data indicates that E_2 does not influence SV40 expression in these cells [9]. The presence of ERβ and ERα in bleo/neo and mp53/neo cells was verified by RT-PCR and ERβ in Western blot (Supplemental Fig. 1). The YAMC bleo/neo and mp53/neo cell lines were created by inserting vectors containing bleomycin alone or bleomycin in conjunction with the *p53*^{175H} mutant gene into YAMC cells as described by Xia and Land [31]. These cell lines were characterized by Xia and Land, notably exhibiting no p53 phosphorylation at Ser15 or acetylation at Lys382 in mp53/neo cells conferring loss of wild type (WT) p53 function in these cells. To explore the role p53 plays in the physiological response of non-malignant colonocytes to E_2 exposure, YAMC bleo/neo and mp53/neo cells were treated with 0, 0.1, 1, or 10 nM E_2 with or without 1 μM ICI, an estrogen receptor antagonist. In cells with WT *p53*, E_2 treatment reduces cell number in a dose dependent manner (Fig. 1A), with an average of a 40% decrease in cell number at the highest dose ($P < 0.01$). The addition of ICI completely abrogates this reduction in cell number. However, in cells containing the *p53* mutant, E_2 treatment had no effect on cell number (Fig. 1B).

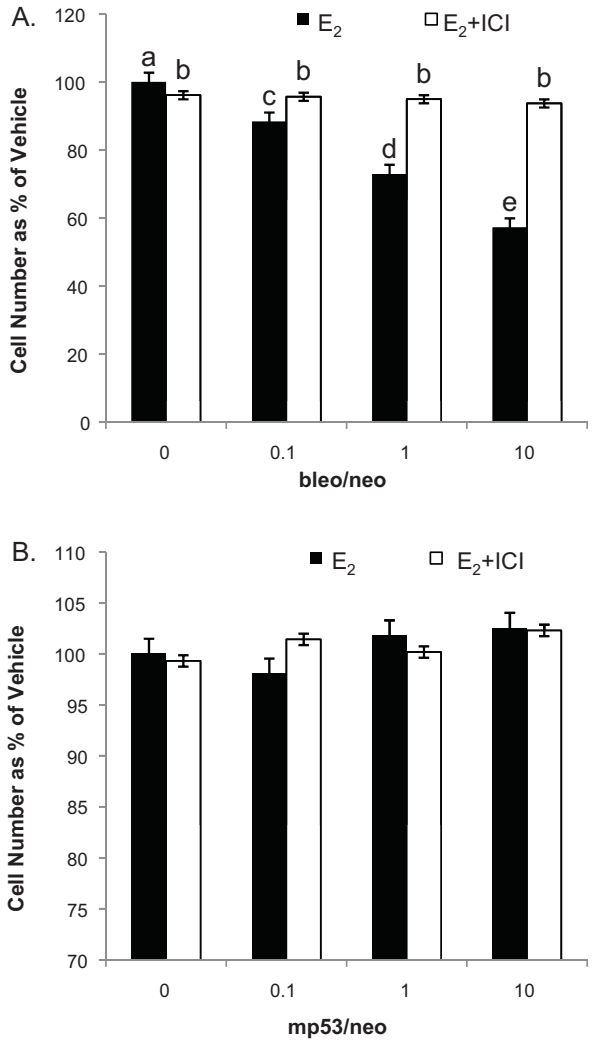

Fig. 1. E_2 inhibits growth of YAMC cells with a functional p53. (A)YAMC bleo/neo cells and B)YAMC mp53/neo cells were grown at 37 °C and in the absence of IFNγ. Cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. 8 h after plating, cells were treated with the listed treatments, media and treatments were replaced 48 h later. After 96 h total treatment time, cells were collected and counted. Data are presented as percentage of vehicle control. Values are means ± SEM, $n = 9$. Bars without a common letter differ, *p*-values <0.01.

3.2. E_2 induced apoptosis requires WT p53

We further investigated p53 activity by studying the role it plays in the induction of apoptosis by E_2. YAMC bleo/neo and mp53/neo cells were treated with 1 nM E_2 or vehicle for 96 h prior to cell lysis. At the end of the treatment period, cells were lysed and a Caspase-3 assay was conducted. The E_2 treated YAMC bleo/neo cells exhibited double the Caspase-3 activity compared to the vehicle treated cells, indicating an increase in apoptosis ($P < 0.001$; Fig. 2A). Conversely, there was no significant difference in Caspase-3 activity between treatments in the YAMC mp53/neo cells (Fig. 2B). Furthermore, the overall degree of apoptotic activity in cells with mutant p53 was approximately one third that observed in the vehicle treated YAMC bleo/neo cells.

3.3. E_2 treatment enhances p53 transcriptional activation

To determine if E_2 treatment alters p53 transcriptional activation, p53 response in the form of a p53-luciferase reporter construct was analyzed. YAMC bleo/neo cells transiently transfected with the PG13-Luc p53 reporter construct and treated with 1 nM E_2 for 24 h exhibited more than a 2.5 fold increase in p53 transcriptional activation compared to cells treated with vehicle alone ($P < 0.01$; Fig. 3).

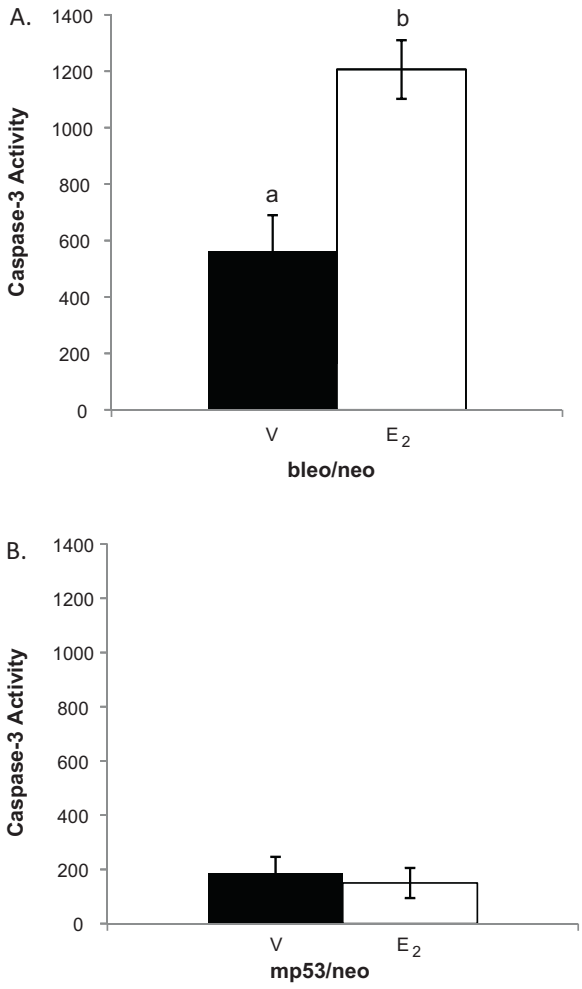

Fig. 2. Functional p53 is necessary for E_2 stimulated apoptosis. (A) YAMC bleo/neo and (B) mp53/neo cells were grown under permissive conditions (33 °C and presence of IFNγ) and transferred to charcoal dextran stripped serum media 48 h prior to plating. After 96 h of 1 nM E_2 treatment, cells were collected and the EnzChek Caspase-3 assay was performed. Data are expressed as relative fluorescence. Values are means ± SEM, $n = 9$. Bars without a common letter differ, p-values <0.001.

As with cell growth, co-treatment with 1 μM ICI completely inhibited E_2 induced increases in p53 transcriptional activation.

3.4. E_2 treatment increases p53 protein while decreasing Mdm2

Having observed increased p53 transcriptional activation, p53 protein levels were analyzed. The amount of p53 protein was measured in cell lysates collected from YAMC bleo/neo cells treated with or without 1 nM E_2 for 0, 4, 8, or 24 h. Cells showed a progressive increase in p53 protein across both treatments, with an overall increase in E_2 treated cells compared to vehicle treatment as time increased, beginning at 4 h, becoming significant at 8 h, with a maximal increase of ~1.5 fold at 24 h ($P < 0.01$; Fig. 4C). However, levels of p53 transcript were not significantly different after 24 h exposure ($P < 0.001$; Fig. 4A). Murine double minute 2 (Mdm2) was also analyzed because of its regulatory relationship with p53, in which Mdm2 binds to inactive p53 and initiates its export from the nucleus and degradation. The transcript levels for Mdm2 were significantly reduced with E_2 treatment (~55% reduction, Fig. 4A) while Mdm2 protein levels were decreased to a lesser degree (~20% reduction, Fig. 4B). These combined data suggest post-transcriptional mechanisms are likely involved in elevating p53 protein concentrations.

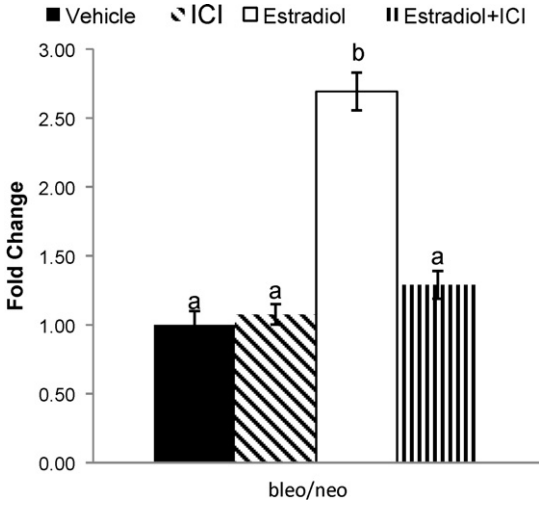

Fig. 3. E_2 enhances p53 transcriptional activity in YAMC cells. YAMC bleo/neo cells were grown at 37 °C and in the absence of IFNγ. Cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. Cells were grown under non-permissive conditions (37 °C and in the absence of IFNγ) for 72 h. After 48 h, cells were transiently transfected with pG13 p53 reporter construct and β-gal construct and treated with vehicle or 1 nM E_2 for the final 24 h. Cell lysates were collected and used to run luciferase and β-galactosidase assays, results were measured on a plate reader. Data are presented as percentage of vehicle control. Values are means ± SEM, $n = 9$. Bars without a common letter differ, p-values <0.01.

3.5. E_2 treatment selectively alters pro-apoptotic p53 target gene expression

Further investigation of p53 function in E_2 treated, non-malignant colonocytes was conducted by analyzing changes in gene expression of p53 down-stream targets. RNA from YAMC bleo/neo cells and mp53/neo cells were collected after treatment with or without 1 nM E_2 for 24 h. Levels of the pro-apoptotic genes Bax, phorbol-12-myristate-13-acetate-induced protein (NOXA) and p53-upregulated modulator of apoptosis (PUMA) were significantly increased following E_2 treatment in YAMC bleo/neo cells ($P < 0.02$; Fig. 5A). The increase in gene expression of Bax was much more pronounced than the changes in other targets. Conversely, the anti-apoptotic gene Bcl-2 transcript was down-regulated ($P < 0.001$). There was no significant change in gene expression for the cell cycle arrest signaling proteins p21 and cyclin dependent kinase inhibitor 1B (p27). When gene expression of these targets were measured under the same treatment conditions in YAMC mp53/neo cells, no change in transcript levels was measured between treatments for Bax, Bcl-2, p21 and p27 ($P < 0.001$; Fig. 5B). However, the gene expression of both NOXA and PUMA were significantly decreased in the E_2 treated cells.

3.6. E_2 treatment reduces radiation induced DNA damage

DNA repair was used as a functional measurement of p53 activity in response to E_2 treatment. Repair of irradiation induced double stranded DNA breaks was analyzed by means of γ-H2AX foci quantification. YAMC bleo/neo and mp53/neo cells were treated for 72 h with 1 nM E_2 or vehicle followed by irradiation. Cells were then stained for γ-H2AX foci and the foci number counted (Fig. 6A–D). Irradiated cells had more than 4 times the number of foci (~56 vs. ~13 in vehicle treatments and ~27 vs. ~5 in E_2 treatments) as those seen in non-irradiated controls ($P < 0.05$; Fig. 6E). Irradiated cells treated with E_2 had a ~50% reduction in the number of observed foci. Similarly, in non-irradiated cells E_2 significantly decreased the number of foci compared to control ($P < 0.001$). In cells without functional p53, E_2 treatment provided no protection against the

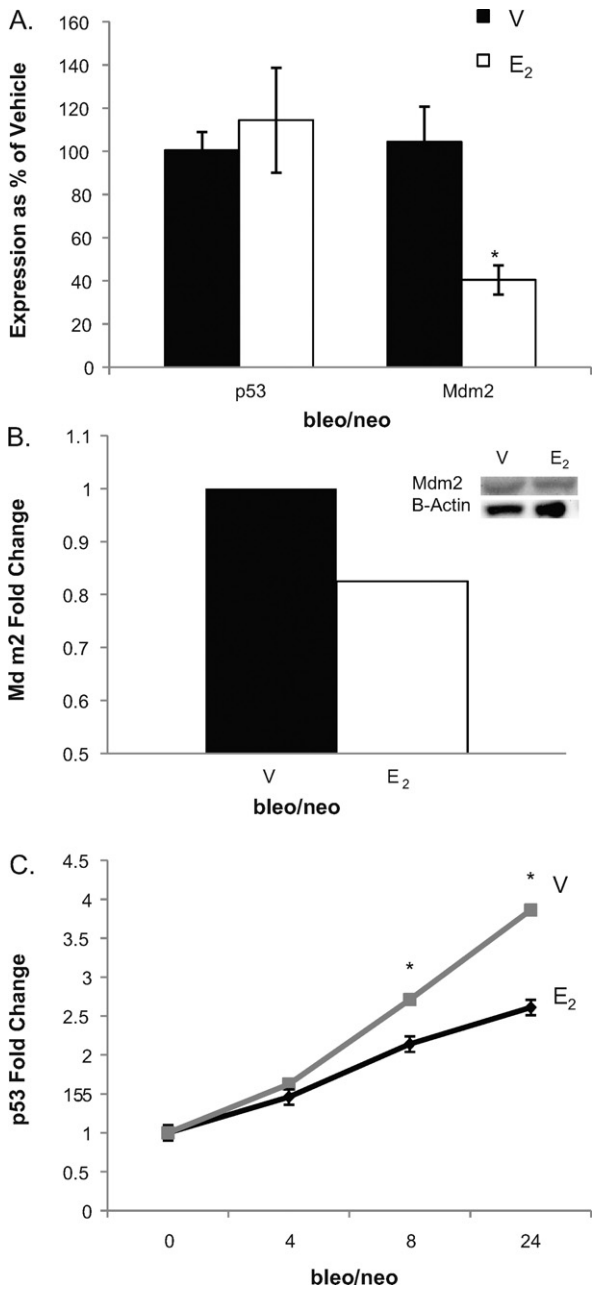

Fig. 4. E_2 increases p53 protein levels, but not transcript levels. YAMC bleo/neo cells were transferred to charcoal dextran stripped serum media 48 h prior to plating. Cells were grown for 72 h under non-permissive conditions (37 °C and in the absence of IFNγ). (A) Cells were treated with vehicle or 1 nM E_2 for the final 24 h, followed by cell lysis and collection of RNA. Two step RT-PCR was conducted using 1 μg RNA for cDNA synthesis and 2 μL cDNA in a 25 μL SYBR green RT-PCR reaction. Data are presented as percentage expression compared to vehicle control. Values are means ± SEM, $n = 9$. Asterisk indicates significant difference, $p < 0.05$. (B) Cells were treated with vehicle or 1 nM E_2 for 24 h, followed by cell lysis and collection of proteins. Western blot using total Mdm2 and β-actin antibodies was then conducted. Data are presented as fold change compared to vehicle treated bleo/neo. Values are means ± SEM, $n = 9$. Asterisk indicates significant difference, $p < 0.05$. (C) Cells were treated with vehicle or 1 nM E_2 for the final 0, 4, 8, or 24 h. At the end of the treatment period cells were lysed and proteins collected. Western blot using a total p53 and β-actin antibodies was then conducted. Data are presented as fold change compared to 0 h vehicle control. Values are means ± SEM, $n = 9$. Asterisk indicates significant difference between treatments at that time point, p-values < 0.015.

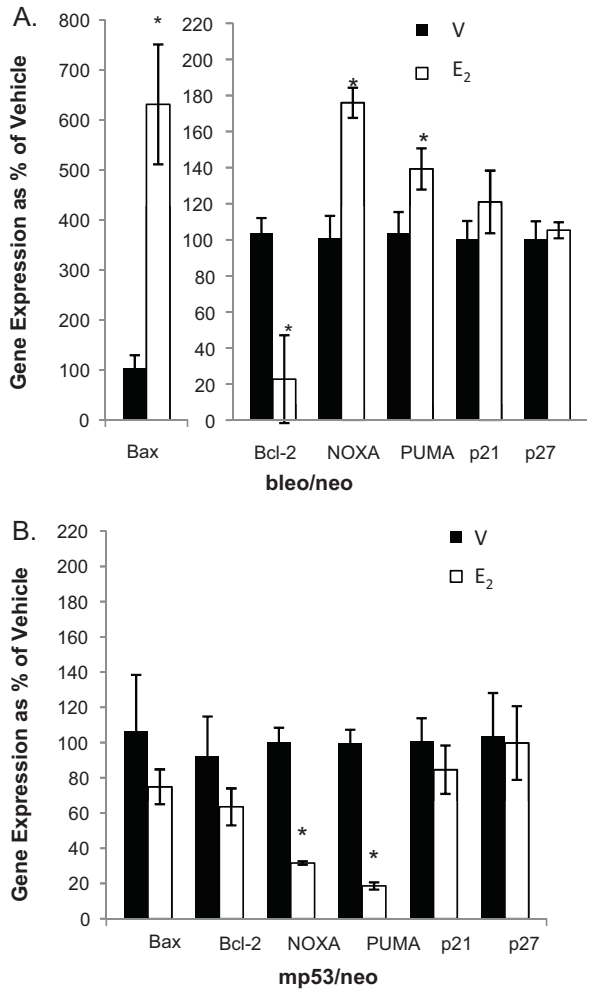

Fig. 5. E_2 alters expression of p53 downstream targets in a pro-apoptotic manner in YAMC cells with a functional p53. (A) YAMC bleo/neo and (B) YAMC mp53/neo cells transferred to charcoal-dextran stripped serum media 48 h prior to plating. Cells were grown under non-permissive conditions (37 °C and in the absence of IFNγ) for 72 h, with vehicle or 1 nM E_2 treatment for the final 24 h. At the end of the treatment time, cells were lysed and RNA collected, and 1 μg total RNA was used to make cDNA. SYBR green based RT-PCR was then conducted using 2 μL cDNA for the listed targets. Values are presented as percentage expression compared to vehicle control treatment ± SEM, $n = 9$. Bars with asterisks differ from vehicle treatment, p-values <0.02.

irradiation damage (data not shown), suggesting wild type p53 is necessary to the pathway involved in E_2 mediated DNA repair in this model.

4. Discussion

The p53 protein is a well studied marker of cancer risk and tumor progression. The loss or mutation of p53 is found in a large percentage of tumors and is often associated with a more severe disease state and poorer prognosis for the patient [32–35]. Many of the *p53* point mutations associated with increased tumor risk are believed to be gain of function in nature, thus offering growth advantages in excess of simple loss of normal p53 function [36]. The $p53^{175H}$ mutant found in the mp53/neo cells is one of the more common point mutations of p53 found in colon cancer [17]. The $p53^{175H}$ mutant is not degraded by Mdm2 like WT p53 allowing it to be the dominant form of the protein in cells carrying this mutant [37]. The p53 protein's role in the initiation of apoptotic pathways in response to DNA damage, aberrant growth signals, or oxidative stress are likely responsible for the decreases in cell

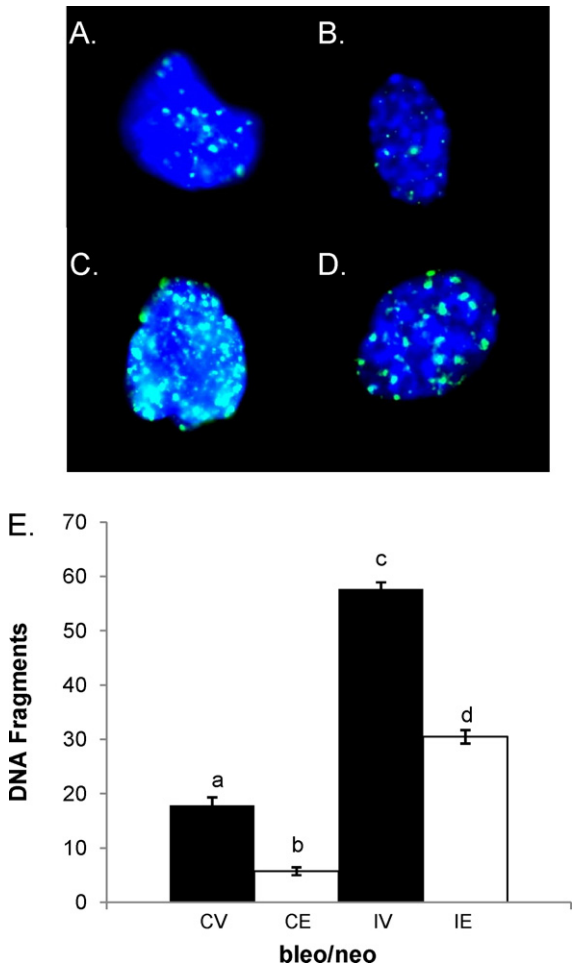

Fig. 6. E_2 enhances repair of radiation induced DNA damage in YAMC cells with a functional p53. YAMC bleo/neo cells were transferred to charcoal-dextran stripped serum media 48 h prior to plating. Cells were grown on 22 mm × 22 mm cover slips in 6-well plates under non-permissive conditions (37 °C and in the absence of IFNγ) for 72 h with vehicle or 1 nM E_2 treatment. At the end of the treatment time, cells were irradiated with 2 Gy followed by incubation at 33 °C for 1 h. Cells were fixed in 4% PFA and cell membrane perforated with 0.3% Triton X-100 in 10% BSA for 1 h. Cells were then incubated overnight with polyclonal anti-phospho γ-H2AX antibody in 10% BSA at 4 °C, followed by incubation with AlexaFluor 488 antibody in 10% BSA for 45 min at room temperature. Cover slips were then treated with ProLong GOLD-DAPI anti-fade solution and mounted on microscope slides. Slides were stored at 4 °C and visualized the next day. Representative pictures for each treatment are shown (A) non-irradiated vehicle (CV), (B) non-irradiated E_2 (CE), (C) irradiated vehicle (IV), and (D) irradiated E_2 (IE). (E) Values are presented as average number of foci observed in 30 cells per slide ± SEM, $n = 9$. Bars without a common letter differ, p-values <0.001.

growth observed in E_2 treated non-malignant colonocytes. Analyses of markers of apoptotic activity shows that YAMC cells require WT p53 activity in order to exhibit E_2 induced apoptosis. We have previously shown that apoptosis is increased in YAMC cells treated with E_2 The introduction of the dominant negative mutant $p53^{175H}$ not only prevents induction of apoptosis by E_2, but significantly down-regulates baseline levels of apoptosis in these cells, highlighting the role of p53 in the regulation of E_2-induced apoptosis. The overall decrease in apoptosis is similar to that found in Hep3B hepatocellular carcinoma cells with the $p53^{175H}$ mutant [38]. This suggests that WT p53 is requisite for E_2 induction of apoptosis in colonic epithelia, and further demonstrates that the $p53^{175H}$ mutation alters the ability of p53 to induce apoptosis within these cells.

Likewise, our data suggests that the ER is important in the influence of E_2 on p53 activity. The primary ER in the colon is ERβ, and we have shown that YAMC cells have upwards of 200 fold higher expression of ERβ than ERα (Supplemental Fig. 1). Our previous data demonstrates that ERβ expression mediates E_2 induced apoptosis in non-malignant colonocytes. Similarly, it was recently shown that over-expression of ERβ in DLD-1 colon adenoma derived cells results in suppression of proliferation when treated with phytoestrogens [39]. It is clear that ER subtypes influence p53 in varying ways. ERα activation leads to export of p53 from the nucleus in MCF-7 cells, however, when ERβ is inserted into these cells p53 activation is up-regulated rather than down-regulated [24]. The ICI abrogation of the E_2 response in bleo/neo cells, which appears to be p53 mediated, suggests that this is an ER dependent event. More compelling is that ICI significantly suppressed E_2 induction of p53 transcriptional activity. Collectively, these data begin to point to one possible mechanism behind the differences in tumorogenicity seen with E_2 exposure between an ERα dominated system, like the breast, compared to an ERβ dominant system, like the colon. Further study using ER subtype specific agonists and antagonists are ongoing to fully determine the role that ERβ plays in this system.

The p53 protein primarily functions by modifying transcriptional regulation of its target genes, such as those promoting apoptosis. There are p53 response elements within the promoter regions of p53 target genes that the DNA-binding region of the p53 protein recognizes and binds to, in order to regulate transcriptional activation [28]. Thus, a p53 response element construct was used in these studies to demonstrate E_2 treatment significantly stimulates the ability of p53 transcriptional activation.

Increased levels of p53 production or increased stabilization of the p53 protein could contribute to this increased p53 transcriptional activation. The lentiviral insertion of ERβ into SW480 colon cancer cells showed increased p53 protein levels with E_2 treatment [40]. Our data demonstrates that p53 mRNA levels are not altered in response to E_2 treatment, indicating that increased p53 production is unlikely. However, increases in p53 protein levels in E_2 treated cells compared to vehicle treated cells suggest that regulation of p53 is taking place at a post-transcriptional level. The basal level of increase in p53 across time points is likely due to the fact that these cells slowly lose viability in cell culture under non-permissive conditions. The amount of p53 protein present in the cells at any time is primarily controlled by the rate of protein degradation [41]. This takes place, in part, through degradation initiated by the ubiquitination of p53. Mdm2 is one of the proteins responsible for this ubiquitination [42,43]. Mdm2 and p53 interact in a regulatory feedback loop in which p53 activates transcription of the Mdm2 gene followed by Mdm2 protein binding p53 and signaling its export from the nucleus and degradation [44]. Thus, p53 in the absence of an activating stimulus will fluctuate slightly, but maintain a relatively stable protein level. Our data demonstrates that E_2 treatment in these cells decreases both Mdm2 transcript and protein levels, further indicating the possibility of increased p53 protein stability as a possible cause of increased p53 protein levels. These data underscore the mechanism of interaction between E_2 treatment and Mdm2 transcriptional regulation as methods of p53 protein regulation and warrant further study.

The integral role of p53 on the ability of non-malignant colonocytes to exhibit a physiological response to E_2 treatment is further supported by gene expression changes in downstream targets of p53. Increased levels of Bax, NOXA, and PUMA are consistent with the previously discussed increases in apoptotic activity, as is the decreased level of Bcl-2, and are indicative of p53 induced apoptosis [45]. The Bcl-2 family proteins Bax, Bcl-2, NOXA, and PUMA are directly involved in the mediation of intrinsic mitochondrial signaling of apoptosis [46]. Bcl-2 is an anti-apoptotic protein that competes with Bax at the mitochondrial membrane to prevent apoptosis. The balance between Bax and Bcl-2 directly affects mitochondrial membrane permeability, and the resultant release of cytochrome-c and triggering of the caspase apoptotic

cascade [47,48]. NOXA and PUMA activate Bax leading to initiation of apoptosis either by direct interaction or indirectly by binding to Bcl-2. The markedly greater increase in Bax:Bcl-2 expression ratio associated with E_2 treatment points to Bax-mediated apoptosis as an important effector pathway of estrogenic chemo-protection in the colon. Decreased Bax:Bcl-2 expression ratio has been associated with tumor progression in AOM induced rat colorectal tumors demonstrating further importance of the Bax:Bcl-2 balance on cancer development [49]. Finally, the transcript levels of p21 and p27, both p53 targets which are associated with cell cycle arrest and senescence, did not change suggesting that E_2-induced p53 activity enhanced apoptosis over senescence. Hartman et al. demonstrated that p21 and p27 protein levels increase in SW480 and HT-29 cells stably expressing exogenous ERβ, however, these cells contain mutant p53 and it is suggested that this is likely responsible for increases in these proteins as the mutated p53 is believed to have lost pro-apoptotic ability [40]. They also demonstrate reduction of p21 protein levels in HCT-116 colon carcinoma cells expressing exogenous ERβ and treated with E_2. In the current study, the majority of the observed changes in gene expression are lost in cells containing the p53 dominant negative mutant. However, increases in NOXA and PUMA expression are not only lost, but are down-regulated in YAMC mp53/neo cells when treated with E_2. This finding might suggest that E_2 can initiate non-p53 dependent negative regulation of these genes, or that E_2 treatment can affect the mutant p53 in a manner other than as a pro-apoptotic stimulus.

Increases in repair of DNA double stranded breaks were also observed illustrating that increased p53 activity in this model is not limited to induction of apoptosis alone. P53 plays a major role in the cellular response to DNA damage and other cellular stresses [50]. Edvardsson et al. demonstrated that E_2 treatment in SW480 cells transfected with an ERβ expression vector increased expression of p53 targets and protected against cell death associated with DNA cross-linkage caused by cisplatin treatment in SW403 cells expressing exogenous ERβ [51]. They also found that double-stranded DNA repair protein, Rad51-like 3 (*S. cervisiae*; RAD51L3), and ribonucleoside-diphosphate reductase subunit M2 B (RRM2B), a p53 target involved in DNA repair, were up-regulated. Conversely, in MCF-7 cells, E_2 activation of ERα inhibits ataxia telangiectasia and RAD3 related (ATR) phosphorylation of p53 in response to DNA damage [52]. The γ-H2AX foci, also known as irradiation induce foci (IRIF), measured in the presented study, identify a specific modification to histone H2A associated with DNA double stranded breaks [53]. The p53 induced gene 3 (PIG3) protein plays an important role in the formation of the γ-H2AX localized MRN repair complex ("fast repair") containing meiotic recombination homolog 11(Mre11), DNA repair protein Rad50 (*S. cerevisiae*) homolog (RAD50) and Nibrin (NBS1) [54]. Furthermore, p53 binding protein 1 (53bp1) recruitment to γ-H2AX foci is enhanced by PIG3 and the absence of p53 greatly reduces PIG3 levels. As such, increased levels of p53 protein and activity, as seen with E_2 treatment in non-malignant colonocytes, may induce DNA repair in the colon. Also of importance, apoptosis itself leads to γ-H2AX foci formation. However, we did not see significant increases in γ-H2AX foci formation even though E_2 induces apoptosis in these cells. The baseline levels of apoptosis in non-malignant colonocytes are low, so the doubling in apoptotic activity we demonstrate would not lead to a large increase in observable foci. The reduction of these foci in irradiated cells treated with 1 nM E_2 suggests that E_2 can induce p53-regulated cellular responses in colonocytes other than apoptosis. Decreases in γ-H2AX foci in non-irradiated cells treated with E_2 suggest that the mechanism(s) involved in E_2 protection/repair of radiation induced DNA damage may be active under normal conditions and significantly up-regulated in response to exposure to radiation, providing the cell with additional protection against DNA damage. This demonstrates another potential role for E_2 in this system. However, these results may be specific radiation-associated DNA damage and future studies will look at other sources of insult to determine this.

In conclusion, the cellular response of non-malignant colonocytes to E_2 treatment relies on the presence of functional p53 protein. Observed phenotypes in cell number changes and induction of apoptosis are lost with the addition of the dominant negative $p53^{175H}$ point mutation. Induction of p53 activity can be observed in response to E_2 exposure along with measurable changes in gene expression levels of p53 downstream targets and stabilization of the p53 protein itself. Finally, E_2-induced repair of DNA damage indicates a potentially broader role of E_2 activity beyond the signaling of programmed cell death due to cellular stress. These findings, when taken together, affirm a role for p53 in E_2 related chemo-protection against colon carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jsbmb.2011.10.010.

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