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PRECLINICAL STUDY

Chronic oxidative stress causes estrogen-independent aggressive phenotype, and epigenetic inactivation of estrogen receptor alpha in MCF-7 breast cancer cells

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Abstract The role of chronic oxidative stress in the development and aggressive growth of estrogen receptor (ER)-positive breast cancer is well known; however, the mechanistic understanding is not clear. Estrogen-independent growth is one of the features of aggressive subtype of breast cancer. Therefore, the objective of this study was to evaluate the effect of oxidative stress on estrogen sensitivity and expression of nuclear estrogen receptors in ER-positive breast cancer cells. MCF-7 cells chronically exposed to hydrogen peroxide were used as a cell model in this study, and their growth in response to 17-β estradiol was evaluated by cell viability, cell cycle, and cell migration analysis. Results were further confirmed at molecular level by analysis of gene expressions at transcript and protein levels. Histone H3 modifications, expression of epigenetic regulatory genes, and the effect of DNA demethylation were also analyzed. Loss of growth in response to estrogen with a decrease in ERα expression was observed in MCF-7 cells adapted to chronic oxidative stress. Increases in *mtTFA* and *NRF1* in these cells further suggested the role of mitochondria-dependent redox-sensitive growth signaling as an alternative pathway to estrogen-dependent growth. Changes in expression of epigenetic regulatory genes, levels of histone H3 modifications as well as significant restorations of both ERα expression and estrogen response by 5-Aza-2′-deoxycytidine further confirmed the epigenetic basis for estrogen-independent growth in these cells. In conclusion, results of this study suggest that chronic oxidative stress can convert estrogen-dependent nonaggressive breast cancer cells into estrogen-independent aggressive form potentially by epigenetic mechanism.

Keywords Oxidative stress · Epigenetics · Breast cancer · MCF-7 cells · Estrogen receptor · Hydrogen peroxide · 5-Aza-2′-deoxycytidine

Introduction

Breast cancer is the most common type of cancer and a leading cause of cancer-related mortality in women [1]. Progression of the breast cancer into more aggressive/metastatic form is the most common factor for mortality in patients [2]. Estrogen receptor (ER)-positive breast cancer is clinically less aggressive and responds to hormone therapy including estrogen antagonists, such as tamoxifen to prevent estrogen-induced growth, and therefore has better prognosis than ER-negative subtype [3, 4]. Hence, ER status is an important indicator of differentiation level in breast cancer, and also as a valuable marker for clinical prognosis. Among two subtypes of ER (ERα and ERβ), role of ERα is considered as critical for breast cancer development and progression, [1, 5, 6]. Even though majority (70–80 %) of the breast cancer arise as ER-positive and less-aggressive types, about 30–40 % of ER-positive tumors will eventually progress into clinically more aggressive form with resistance to hormonal therapy [3, 7].

Multiple mechanistic pathways have been proposed for progression of ER-positive breast cancer into more aggressive phenotype, which includes loss of ER expression [8], phosphorylation of serine residues in ERα through signaling pathways mediated by over expression of

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epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) [9–11], activation of kinase pathway leading to phosphorylation of ER related accessory proteins [12], miRNA-mediated (miRNA-221/222) regulation of ERα [13, 14], and redistribution of estrogen receptors within the cell [15]. Among all these different mechanisms, the decrease or loss of ERα expression in breast cancer is considered as the most important factor for progression of breast cancer cells into more aggressive form and for developing resistance to hormone therapy [1, 16]. However, the mechanistic understanding of factors driving the nonaggressive ER-positive breast cancer into aggressive form is still unclear.

Oxidative stress-mediated signaling and anti-oxidative response pathways are active during breast carcinogenesis, and these pathways have been positively correlated with progression of disease as well as clinical prognosis in breast cancer patients [17]. Multiple reports showing higher level of reactive oxygen species (ROS) in more aggressive and metastatic breast cancer cells than primary tumor cells, and potential use of antioxidant compounds to reduce tumor metastasis further provide strong evidence for the role of oxidative stress in breast cancer progression [18, 19]. We have recently reported that chronic exposure to oxidative stress leads to increased growth and tumorigenic potential of ERα-positive MCF-7 breast cancer cells [20]. In addition, accumulating evidence from both epidemiological and experimental studies indicate increased levels of ROS leading to oxidative stress burden in breast cancer cells. For example, increased levels of oxidative stress injury markers have been reported in both serum [21] and urine [22] samples of breast cancer patients.

Even though there is no evidence to support the direct involvement of oxidative stress in regulation of ER status of breast cancer cells, several studies suggest a correlation between the level of oxidative stress marker and expression and/or activity of ER. For example, increased expression of anti-oxidative response genes and functional loss of ER represent poor prognosis in ER-positive breast cancer cells [23]. Estrogen-induced phosphorylation of ERα may also be regulated by ROS via AKT, because co-treatment of estrogen with the ebselen (20 μM), a potent scavenger of H_2O_2, hydroperoxides, and peroxynitrite, can decrease estrogen-induced phosphorylation of ERα in MCF-7 cells [24]. Redox-sensitive transcription factor NF-kβ is also known to regulate expression level of ER through multiple mechanisms [25]. Activation of c-Jun N-terminal kinase (JNK) by chronic oxidative stress has frequently been reported. Recently, acquisition of ER-independent proliferative mechanism through constitutive activation of JNK due to its decreased phosphorylation by Insulin-like growth factor (IGFR) has been reported in MCF-7 cells [26]. These reports suggest a potential role of oxidative stress in increasing the aggressiveness in ER-positive breast cancer cells; however, the effects of oxidative stress on estrogen sensitivity and ER expression in estrogen-responsive breast cancer cells are not known.

In this context, the objective of the current study was to evaluate whether the acquired aggressiveness in ER-positive MCF-7 breast cancer cells exposed to chronic oxidative stress is mediated by the modulation of estrogen sensitivity and ER expression. In addition, based on several recent reports showing DNA hypermethylation as a frequently observed mechanism for silencing of ERα in ER-negative breast cancer cells [27–29], we further evaluated the potential role of epigenetic mechanism in oxidative stress-induced alterations in estrogen sensitivity, ER expression, and acquired aggressive phenotype in MCF-7 cells chronically exposed to oxidative stress.

Materials and methods

Chemicals and reagents

Hydrogen peroxide (H_2O_2) solution (30 % w/v), 2′,7′-dichlorodihydrofluorescindiacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 17-β estradiol, tamoxifen, and 5-Aza-2′-deoxycytidine (5-aza 2′ dC) were purchased from Sigma (St. Louis, MO). Quantitative real-time PCR reagents were purchased from BioRad, Inc., and Guava cell cycle reagent was obtained from Millipore (Hayward, CA). RIPA lysis buffer (1X) for protein isolation was purchased from Santa Cruz Biotechnology, Inc. Phenol red-free DMEM medium, trypsin/EDTA solution, Fetal Bovine Serum (FBS), antibiotic/antimycotic solution, and Trizol reagent (for RNA isolation) were procured from Invitrogen Inc. (Carlsbad, CA).

Cell culture and treatment

MCF-7 cells (ER-positive breast cancer cell lines) and MDA-MB-231 cells (ER-negative breast cancer cell lines) were purchased from American Type Culture Collection (ATCC). Cells were grown and maintained in phenol red-free DMEM medium supplemented with 10 % Fetal Bovine Serum and 1 % solution of antibiotic and antimycotic in an incubator at 37 °C with 5 % CO_2 in a humidified atmosphere. To develop chronic oxidative stress cell model, MCF-7 cells were exposed to noncytotoxic (25 μM) and significantly cytotoxic (250 μM) doses of hydrogen peroxide chronically for 6 months. Exogenous hydrogen peroxide is known to induce increased intracellular ROS [30, 31], hence commonly used to induce oxidative stress in in vitro models. In brief, actively

growing MCF-7 cells were seeded in 25-cm^2 cell-culture flasks and treated with H_2O_2 at 60-70 % confluence in fresh culture media. H_2O_2-exposed MCF-7 cells, were subcultured (after reaching approximately 80 % confluency), grown for 24 h, and then given fresh H_2O_2 treatment. Using this method, MCF-7 cells were exposed to chronic oxidative stress by growing them in H_2O_2 containing media for 6 months. Parallel cultures of MCF-7 cells were grown in H_2O_2-free media and maintained as passage-matched controls.

To evaluate the effect of chronic oxidative stress on estrogen-induced proliferative response and tamoxifen-induced growth inhibitory response, MCF-7 cells exposed to chronic oxidative stress were treated with 17-β estradiol (100 pg/ml) and tamoxifen (1 μM) for 48 h. To further evaluate the role of epigenetic alterations, MCF-7 cells exposed to chronic oxidative stress were first pretreated with demethylating agent (2 μM 5-aza 2′ dC) for 24 h and subsequently co-treated with 5-aza 2′ dC and 17-β estradiol or tamoxifen for 48 h. Similar treatment schedule was followed for MDA-MB-231 breast cancer cells, which were used as a positive control for ER-negative breast cancer cells. Vehicle control groups of MCF-7 and MDA-MB-231 cells were treated with DMSO at a final concentration of 0.001 % in their culture medium.

MTT assay for cell growth and viability

To evaluate growth response of chronic oxidative stress adapted MCF-7 cells to 17-β estradiol and tamoxifen, MTT dye reduction assay was performed. ER-positive MCF-7 cells exposed to 25 μM and 250 μM H_2O_2 and ER-negative MDA-MB-231 cells were seeded (2000 cells per well) in 96-well cell-culture plates and allowed to attach and grow for 24 h. Cells were treated with 17-β estradiol (100 pg/ml) and tamoxifen (1 μM) with and without 5-aza 2′ dC as mentioned earlier and then MTT solution (10 mg/mL in PBS) was added to each well and incubated at 37 °C with 5 % CO_2 for 3 h. To dissolve insoluble formazan crystals thus formed by mitochondrial activity of viable cells, culture media was completely removed and DMSO (150 μL) was added to each well,. The color intensity in each well was measured using microplate reader at 570- and 630-nm absorbances. Each treatment was performed in triplicates, and experiment was repeated twice. Passage-matched untreated MCF-7 and MDA-MB-231 cells were used as control.

Cell cycle analysis by flow cytometry

Effects of chronic oxidative stress on estrogen response were also confirmed by evaluating cell cycle changes following 17-β estradiol exposure. MCF-7 cells (500,000 cells) chronically exposed to H_2O_2-induced oxidative stress and their passage-matched untreated control cells were seeded in 25-cm^2 cell-culture flasks. Cells were treated with 17-β estradiol for 48 h as described earlier under treatment section, and cells were used for cell cycle analysis. To evaluate role of DNA methylation, MCF-7 cells exposed to chronic oxidative stress were treated with 5-aza 2′ dC for 24 h before 17-β estradiol treatment and also co-treated with 17-β estradiol for 48 h. Control group cells were treated with DMSO as a vehicle control. Actively growing cells were collected after 48 h of treatment and fixed using 70 % ethanol for 24 h at 4 °C. Fixed cells were stained with Guava cell cycle reagent (Millipore), and cell cycle changes were analyzed by counting 5000 cells (events) in Guava Easy-Cyte HT Flow Cytometer (Millipore). Guava Incyte software (Millipore) was used for analysis of cell cycle data. All samples were analyzed in triplicates, and experiment was also repeated twice.

Scratch wound healing assay for migration potential

To evaluate the migration potential of MCF-7 cells exposed to H_2O_2-induced oxidative stress for chronic period, wound healing assay was performed. Actively growing MCF-7 cells exposed to 25 and 250 μM H_2O_2 for 6 months, and passage-matched control cells were seeded into six-well cell-culture plates and allowed to grow for 48 h. Further, to evaluate role of epigenetic mechanism, cells in each group exposed to demethylating agent were also seeded in six-well plates. At 50–60 % confluence, three parallel scratch wounds were made in each well using sterile plastic micropipette (200 μl) tips. After creating scratch wounds, cell-culture media were replaced with fresh media to remove floating cells or debris, and the plates were incubated at standard cell-culture conditions. Plates were observed daily for migration of cells into wounded area, and representative photomicrographs were taken at days 0, 2, and 4.

Extraction of RNA and quantitative real-time PCR

Total RNA was extracted from actively growing MCF-7 cells exposed to chronic oxidative stress and their passage-matched control cells using Trizol reagent. Genes of interest were amplified by MyiQ2 real-time PCR detection system (BioRad Laboratories, Hercules, CA) using SYBR green one-step RT-PCR kit and 200 ng total RNA following procedure described in manufacturer's protocol (Bio-Rad). PCR reactions were performed in triplicates using primers specific for *ERα, NRF-1, mtTFA* and epigenetic regulatory genes (*DNMT1, DNMT3b, MBD4, HAT1*, and *HDAC1*). Thermal cycling in RT-PCR machine was programmed for reverse transcription at 50 °C for 15 min,

denaturation and inactivation of reverse transcriptase enzyme at 95 °C for 5 min, followed by 40 cycles each containing 10 s at 95 °C for denaturation and 30 s for annealing and extension at 60 °C. PCR data of each gene were normalized to housekeeping gene (GAPDH) cycle threshold (Ct) value obtained from the same sample and the delta–delta C_t method [32] was used to calculate fold changes in gene expression. List of primer sequences used for analysis of changes in gene expression have been listed in Table 1.

Immunoblotting analysis

Total proteins were extracted from MCF-7 cells exposed to chronic oxidative stress and their passage-matched control cells using RIPA lysis buffer. Protein samples were electrophoresed on a 10 % SDS-PAGE gel and then transferred to nitrocellulose membranes. Membranes were incubated with 5 % nonfat-dried milk for 1 h at room temperature, to block nonspecific binding sites, and then probed with diluted primary antibody overnight at 4 °C. To achieve better reaction efficiency, primary antibodies with dilution of 1:1000 of *ERα* (Cell signaling, part of Cat #9924), 1:500 of *NRF-1* (Santa Cruz, Cat #sc33771), 1:100 of *DNMT1* (Santa Cruz, Cat#10219), 1:100 of *MBD4* (Santa Cruz, Cat #sc-10754), 1:1000 of *Acetyl H3K9* (Cell signaling, Cat #9671), 1:1000 of *Acetyl H3K18* (Cell signaling, Cat #9441) and 1:250 of *GAPDH* (Santa Cruz, Cat #sc-25778) were used. Molecular weight markers were also run on gels to compare protein size. After washing the membranes in washing buffer, incubated with diluted (1:1000) horseradish peroxidase conjugated secondary antibody in room temperature for 1 h. Immuno-reactive bands were visualized by using an enhanced chemiluminescence detection method (Amersham, NJ). The signal intensity of protein bands were quantified using software (Image J) and also normalized to expression of housekeeping gene (GAPDH). The signal intensity of proteins in different treatment groups were converted to fold changes compared to control, by considering signal intensity in control as 1-fold.

Statistical analysis

Two tailed *t* test (paired samples for means) was performed to evaluate statistical significance of the changes observed in multiple parameters. Further an analysis of variance (ANOVA) was performed to ascertain whether the source of variation in the data was intragroup or intergroup differences. Level of significance (α) was set at 0.05 and differences with $p < 0.05$ were considered as statistically significant.

Results

To evaluate the effects of chronic oxidative stress on estrogen response and ER expression in ER-positive breast cancer cells, MCF-7 cells were exposed to H_2O_2-induced oxidative stress for 6 months and then used for evaluation at different levels. To further understand the possible role of epigenetic changes, chronic oxidative stress-exposed MCF-7 cells were treated with demethylating agent (5-aza 2′ dC) and then used for analysis of estrogen response.

Effect of chronic oxidative stress on estrogen response as evaluated by MTT assay

To evaluate the effect of chronic oxidative stress on estrogen-induced proliferative response and anti-estrogen drug-induced cell growth inhibition, MCF-7 cells were exposed to 17-β estradiol (E2) and anti-estrogen (Tamoxifen) as described in method section and cell growth/viability was measured by MTT assay. Results of MTT assay for E2 response and tamoxifen response are given in Figs. 1 and 2, respectively.

Exposure of E2 resulted in statistically significant (63 %) proliferative response in control group MCF-7

Table 1 List of primer sequences for genes analyzed by real-time quantitative PCR

Gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Size (base pair)
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT	116
NRF1	GTCCAGATCCCTGTGAGCAT	GGTGACTGCGCTGTCTGATA	117
mtTFA	TATCAAGATGCTTATAGGGC	CACTCCTCAGCACCATATTTTCG	430
ERα	GAATCGCCAAGGAGACTCG	ATCATCTCTCTGGCGCTTGT	291
DNMT1	GTGGGGGACTGTGTCTCTGT	GAAAGCTGCATGTCCTCACA	115
DNMT3b	ACCAGTGGTTAATAAGTCGAAGG	CTCGGCTCTGATCTTCATCCC	143
MBD4	CAGGCAAAATGGCAATACCT	GTTTTTGCCCGAAGCTCGTA	184
HDAC1	TGGAAATCTATCGCCCTCAC	TCTCTGCATCTGCTTGCTGT	128
HAT1	GGTGATTCGTCCTTCCTCA	GCCAGTTTCTTCTCCACTGC	112

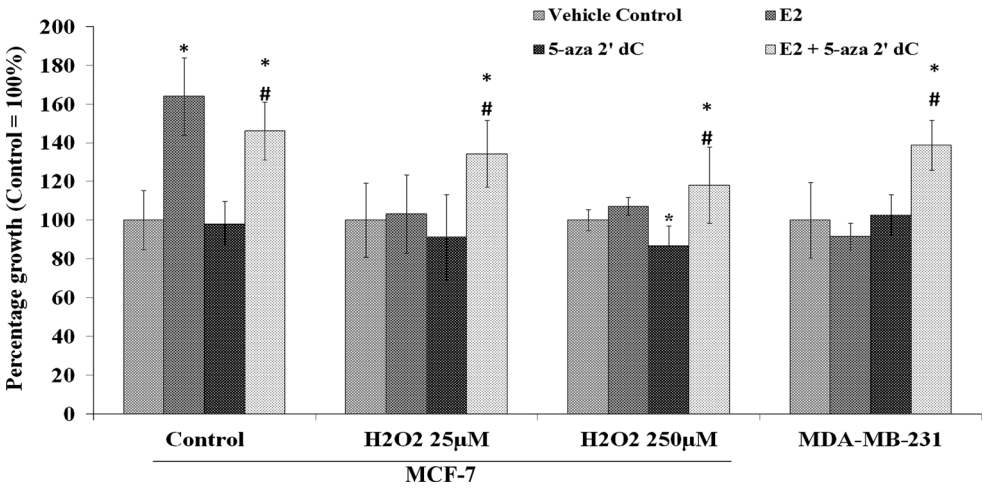

Fig. 1 *Bar graph* showing the mean value of cellular viability in response to E2 treatment. MCF-7 cells adapted to chronic oxidative stress were exposed to 17-β estradiol (100 pg/ml) for 48 h, and their growth response was analyzed by MTT assay as described in "Materials and methods" section. Similarly, to evaluate the effect of demethylation on estrogen-induced growth response, cells were pretreated with 5-aza 2′ dC for 24 h and then exposed to 17-β estradiol. MDA-MB-231 cells were used as a positive control representing ER-negative breast cancer cells. The *error bars* represent the standard deviation of the mean (±SD). An *asterisk* indicates statistically significant ($p < 0.05$) change, compared to vehicle control. Symbol *hash* indicates statistically significant ($p < 0.05$) change, compared to respective E2-treated group without 5-aza 2′ dC treatment

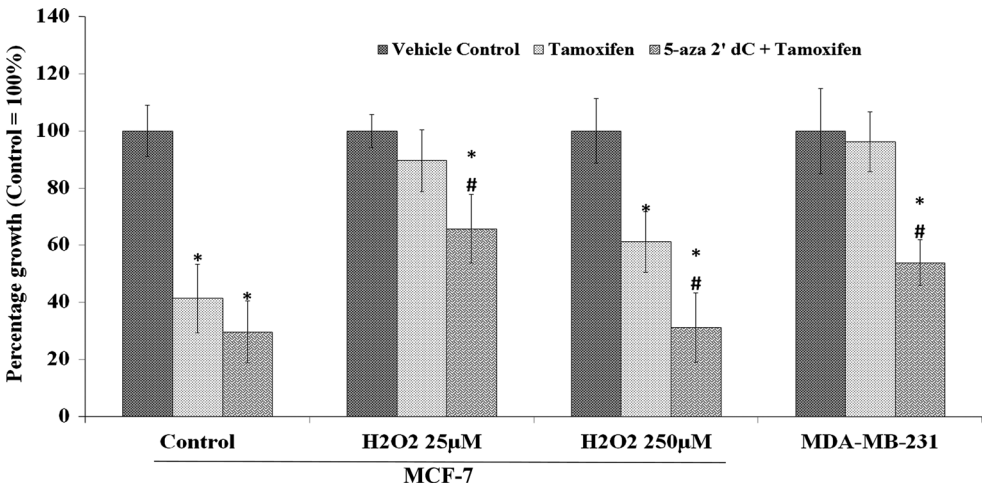

Fig. 2 *Bar graph* showing the mean value of cellular viability in response to tamoxifen treatment. MCF-7 cells adapted to chronic oxidative stress were exposed to tamoxifen (1 µM) for 48 h and cell growth response was analyzed by MTT assay as described in "Materials and methods" section. Further, to evaluate the effect of demethylation on tamoxifen-induced growth inhibition, cells were pretreated with 5-aza 2′ dC for 24 h and then exposed to tamoxifen. MDA-MB-231 cells were used as a positive control representing ER-negative breast cancer cells. The *error bars* represent the standard deviation of the mean (±SD). An *asterisk* indicates statistically significant ($p < 0.05$) change, compared to vehicle control. Symbol *hash* indicates statistically significant ($p < 0.05$) change, compared to respective tamoxifen-treated group without 5-aza 2′ dC treatment

cells, whereas no significant increase in cell growth were observed in MCF-7 cells adapted to 25 µM and 250 µM H_2O_2 (Fig. 1). As expected, ER-negative MDA-MB-231 cells didn't show any significant growth response to E2 treatment. Hence, estrogen response in chronic oxidative stress-exposed MCF-7 cells was similar to ER-negative MDA-MB 231 cells. Similarly, significant decrease in response to antiestrogen (tamoxifen) was observed in MCF-7 cells exposed to chronic oxidative stress, compared to passage-matched control cells. In control MCF-7 cells, 58.64 % decrease in cell growth was observed following tamoxifen treatment, whereas, only 10.33 and 38.82 % decrease in cell growth was observed in MCF-7 cells exposed to 25 and 250 µM H_2O_2, respectively (Fig. 2). This change was statistically significant only in 250 µM H_2O_2 group. Response to tamoxifen in 25 µM H_2O_2-

treated group was not statistically significant, compared to its concurrent control and was comparable to growth inhibition observed in ER-negative MDA-MB-231 cells.

To further evaluate the effect of demethylation on the chronic oxidative stress-induced changes, cells were exposed to 5-aza 2′ dC as mentioned in methods section and then evaluated for cell growth response to E2 and tamoxifen. 5-aza 2′ dC treatment alone did not show any significant cytotoxicity in either control MCF-7 cells or in cells exposed to chronic oxidative stress. Whereas, improvement in sensitivity of chronic oxidative stress adapted MCF-7 cells to both E2- induced proliferative response and tamoxifen-induced growth inhibition was observed following 5-aza 2′ dC treatment. Statistically significant increase in E2-induced growth by 34 and 17 % was observed in 25 and 250 μM H_2O_2 group, respectively, compared to untreated control (Fig. 1). This increase was about 30 % in 25 μM and by 10 % in 250 μM H_2O_2 cells, compared to respective groups without 5-aza 2′ dC exposure. Similarly, 5-aza 2′ dC treatment significantly increased tamoxifen-induced cell growth inhibition in both 25 μM and 250 μM H_2O_2 group by 34.29 and 38.82 %, respectively, compared to control cells. The increase in tamoxifen response following 5-aza 2′ dC treatment was highest (73 %) in 25 μM H_2O_2-exposed cells, compared to respective group without 5-aza 2′ dC exposure (Fig. 2). ER-negative MDA-MB-231 cells also showed significant increase to both E2-induced cell proliferation (38.70 %) and tamoxifen-induced cell growth inhibition (46.11 %) following treatment with demethylating agent. These results indicated that adaptive response to chronic oxidative stress significantly decreased E2 and tamoxifen response in MCF-7 cells and 5-aza 2′ dC treatment was able to significantly restore the response.

Effect of chronic oxidative stress on estrogen-induced cell cycle changes

Chronic oxidative stress-induced decrease in response to estrogen was also confirmed by evaluating cell cycle changes in MCF-7 cells after 17-β estradiol treatment. E2 is known to induce proliferative response in ER-positive MCF-7 breast cancer cells. Hence, in this study we were mainly interested in evaluating changes in S phase of cell cycle following E2 exposure of MCF-7 cells adapted to chronic oxidative stress. The results of cell cycle analysis and the percentage of cells in S phase in each treatment group are given in Fig. 3a, b. As expected, exposure of control group MCF-7 cells to 100 pg/ml E2 for 48 h significantly increased (72.28 %) percentage of cells in S phase of the cell cycle. But in MCF-7 cells chronically exposed to 25 μM and 250 μM H_2O_2, there was no significant increase in percentage of S phase cells following E2 treatment (Fig. 3b). These results of cell cycle analysis further confirmed decreased response to E2 in MCF-7 cells adapted to both low and high dose of chronic oxidative stress. Similar to MTT results, cell cycle data also confirmed significant increase in cell growth response to E2 following pretreatment and co-treatment of 5-aza 2′ dC with E2 in chronic oxidative stress-exposed MCF-7 cells. For example, 5-aza 2′ dC exposure resulted in statistically significant increase is percentage of S phase cells by 21 and 27.5 % in MCF-7 cells exposed to 25 and 250 μM H_2O_2, respectively. The results of cell cycle analysis also suggested hyper-methylation-mediated mechanism in regulating chronic oxidative stress-induced changes in estrogen response.

Effect of chronic oxidative stress on migration potential

To evaluate the effect of chronic oxidative stress on migration potential of MCF-7 cells, wound healing scratch assay was performed. Microscopic evaluation of migration of cells into scratch wound area at different intervals showed increased migration potential of MCF-7 cells adapted to chronic oxidative stress (Fig. 4). MCF-7 cells in both 25 and 250 μM H_2O_2 treatment groups migrated early into wound area compared to untreated passage-matched control cells. When compared within the treatment groups, MCF-7 cells adapted to 25 μM H_2O_2 showed visibly increased migration potential than cells adapted to 250 μM H_2O_2 at all-time points evaluated (Fig. 4). In 5-aza 2′ dC-treated groups, migration potential of MCF-7 cells was comparable in all the groups, indicating decreased migration of chronic oxidative stress adapted MCF-7 cells with 5-aza 2′ dC treatment.

Effect of chronic oxidative stress on gene expression changes at transcript level

To further understand the molecular mechanisms associated with adaptive response to chronic oxidative stress leading decreased growth response to E2 and tamoxifen in ER-positive MCF-7 breast cancer cells, changes in expression of estrogen receptor (ERα), genes involved in mitochondrial activity (*mtTFA* and *NRF-1*) and epigenetic regulatory genes (*DNMT1, DNMT3b, MBD4, HAT1*, and *HDAC1*) were evaluated by quantitative real-time PCR analysis. The changes observed in each category of genes are as follows.

Changes in expression of ER gene

Expression of *ERα* was evaluated at transcript level in MCF-7 cells adapted to chronic oxidative stress. Significant downregulation of *ERα* expression was observed in

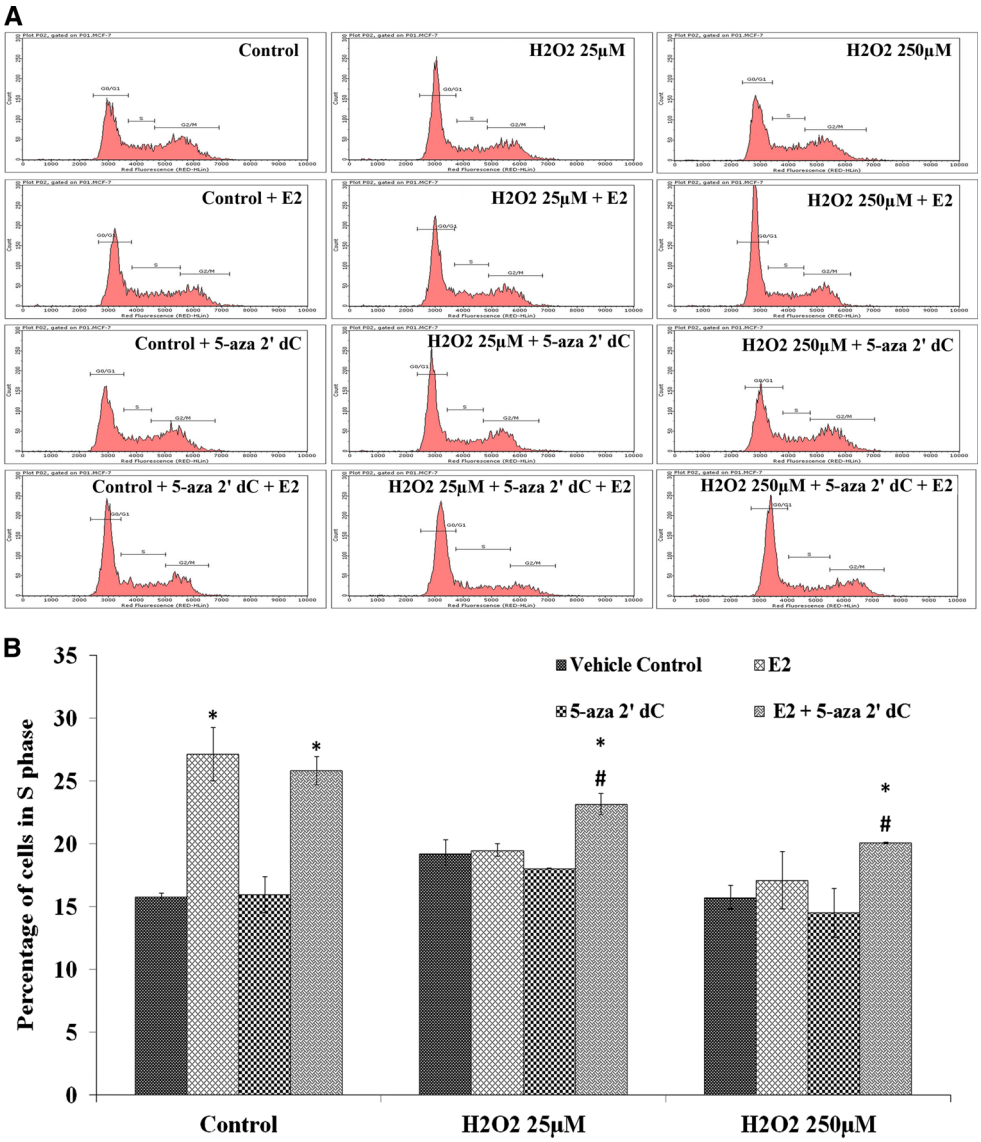

Fig. 3 Representative histograms of cell cycle analysis by flow-cytometry (**a**), and bar graph (**b**) showing percentage of cells in S phase of cell cycle following E2 exposure with and without 5-aza 2′ dC pretreatment in MCF-7 cells adapted to chronic oxidative stress. Cells were treated, collected, fixed, and stained for cell cycle analysis by flow-cytometer by following the standard procedure as described in "Materials and methods" section. Percentage of cells in S phase of cell cycle represents the average value from three independent cell cycle analysis experiments. The *error bars* represent the standard deviation of the mean (±SD). An *asterisk* indicates statistically significant ($p < 0.05$) change, compared to vehicle control. Symbol *hash* indicates statistically significant ($p < 0.05$) change, compared to, respective group without 5-aza 2′ dC treatment

both low and high dose oxidative stress adapted cells. *ERα* level was down regulated by -4.28- and -3.71-folds in MCF-7 cells exposed to 25 and 250 µM H_2O_2, respectively (Fig. 5a). 5-Aza 2′ dC treatment significantly restored *ERα* expression in both 25 and 250 µM H_2O_2-exposed cells, compared to untreated cells. In 25 µM H_2O_2 group, 5-aza 2′ dC treatment restored *ERα* expression from −4.28-fold to −2.04-folds. Similarly in 250 µM H_2O_2 group, *ERα* transcript level changed from −3.71-folds to −1.56-fold, compared to passage-matched control cells (Fig. 5a).

Changes in expression of genes involved in mitochondrial activity

Representative genes associated with mitochondrial activity such as mitochondrial transcription factor A (*mtTFA*) and its regulator Nuclear respiratory factor-1 (*NRF1*) were evaluated at transcript level. Significant increase in expression of both *mtTFA* and *NRF-1* were observed in MCF-7 cells adapted to chronic oxidative stress. In 25 µM H_2O_2-exposed group, increase by 1.89- and 3.23-folds was observed in *mtTFA* and *NRF-1*, respectively (Fig. 5a). Similarly in 250 µM H_2O_2-exposed group, both *mtTFA* and *NRF-1* were upregulated by 1.35- and 2.05-fold, respectively. 5-aza 2′ dC treatment induced further upregulations of both *mtTFA* and *NRF-1* in cells adapted to 25 µM and 250 µM H_2O_2. *mtTFA* was upregulated by 2.01-fold and 1.84-fold, respectively, in 25 and 250 µM H_2O_2-exposed group, following 5-aza 2′ dC treatment. Similarly, transcript expression level of *NRF-1* was significantly increased by 2.80-fold and 3.60-fold in 25 and 250 µM H_2O_2-exposed groups, respectively (Fig. 5a).

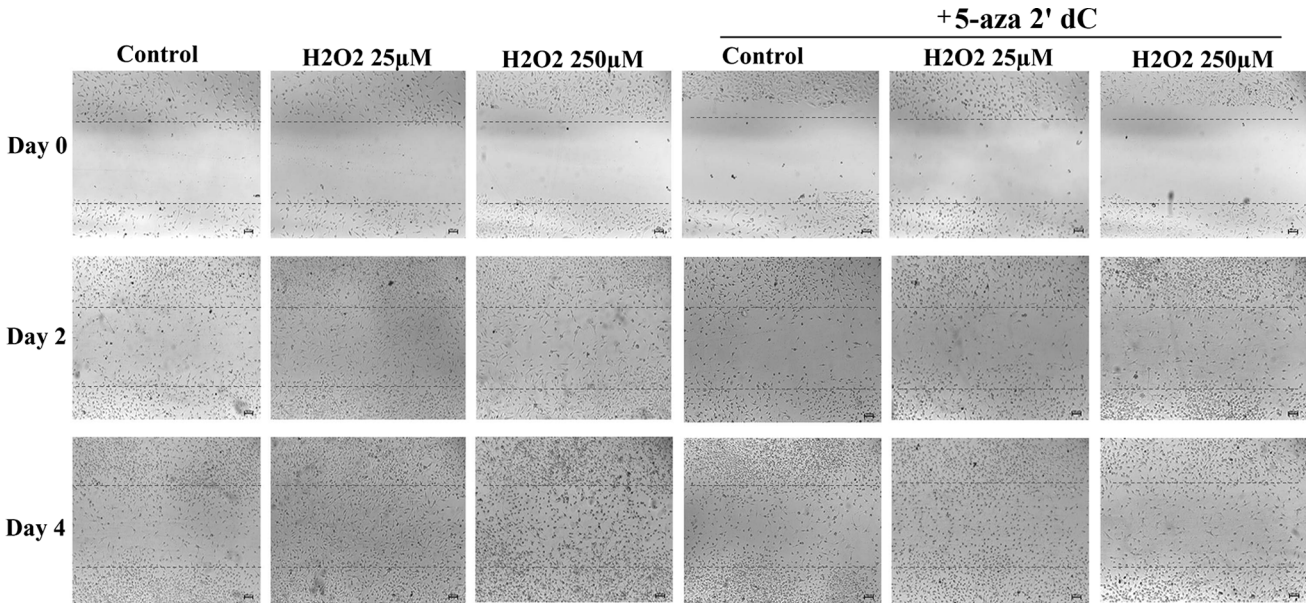

Fig. 4 Representative photomicrographs (×40 magnification) showing migration potential of MCF-7 cells adapted to chronic oxidative stress. MCF-7 cells after chronic exposure to oxidative stress were seeded in six-well plates and then 5-aza 2′ dC treatment and wound healing scratch assay was performed as described in "Materials and methods" section. Each well in plates were microscopically observed daily to evaluate migration of cells into wounded area and representative photomicrographs were taken at Day 0, 2 and 4. *Dotted lines* on the photomicrographs indicate wound edges

Changes in expression of epigenetic regulatory genes

To further understand the role of epigenetic regulation in chronic oxidative stress-induced changes, representative genes involved in DNA methylation (*DNMT1, DNMT3b and MBD4*) and histone modifications (*HAT1* and *HDAC1*) were evaluated at transcript level. Significant increase in expression of enzymes involved in transfer of methyl group to DNA (*DNMT1* and *DNMT3b*) as well as a methyl-binding protein (*MBD4*) were observed in MCF-7 cells chronically exposed to both 25 and 250 μM H_2O_2 (Fig. 6). Significant increase in *DNMT1, DNMT3b* and *MBD4* expression by 2.69-, 3.08-, and 2.19-folds, respectively was observed in MCF-7 cells adapted to 25 μM H_2O_2. In 250 μM group, expression of DNMT1, DNMT3b and *MBD4* were upregulated by 4.03-, 2.45-, and 3.09-folds, respectively (Fig. 6). Similarly, downregulation of *HAT1*, an enzyme involved in transfer of acetyl group to histones and upregulation of *HDAC1,* an enzyme involved in removal of acetyl group from histones was observed in both 25 and 250 μM H_2O_2-exposed cells. In 25 μM H_2O_2 group, *HAT1* expression was downregulated by −1.89-fold (statistically insignificant), whereas *HDAC1* expression upregulated by 3.07-folds (statistically significant). In 250 μM H_2O_2 group, expression of *HAT1* was significantly downregulated by −2.57-folds and expression of *HDAC1* was significantly upregulated by 5.33-folds.

Following treatment of cells with DNA-demethylating agent (5-aza 2′ dC), significant reversal of changes observed in expression of *DNMT1, DNMT3b*, and *MBD4* was observed. 5-aza 2′ dC treatment of MCF-7 cells exposed to 25 μM H_2O_2 resulted in −1.94, −2.54 and −1.21-fold decrease in *DNMT1, DNMT3b*, and *MBD4* expressions, respectively (Fig. 6). Similarly, in 250 μM H_2O_2 group, 5-aza 2′ dC treatment downregulated expressions of *DNMT1, DNMT3b*, and *MBD4* by −2.33, −1.66, and −1.78-folds, respectively. All these gene expression changes observed after 5-aza 2′ dC treatment were statistically significant in both 25 and 250 μM H_2O_2 group, compared to passage-matched control cells. In addition, changes in both *HAT1* and *HDAC1* were also restored to some extent without statistical significance following 5-aza 2′ dC treatment. In 25 μM H_2O_2 group, expression levels of *HAT1* and *HDAC1* were changed from −1.89 and 3.07-folds to 1.21 and 1.45-folds, respectively, after 5-aza 2′ dC treatment. Similarly, in 250 μM H_2O_2 group, 5-aza 2′ dC treatment resulted in change in expression levels of *HAT1* and *HDAC1* from −2.57- and 5.33-folds to −1.34 and 2.32-folds, respectively.

Effect of chronic oxidative stress on protein expression and histone modifications

Immunoblot analysis was performed to confirm the transcript level changes observed in *ERα, NRF-1*, and

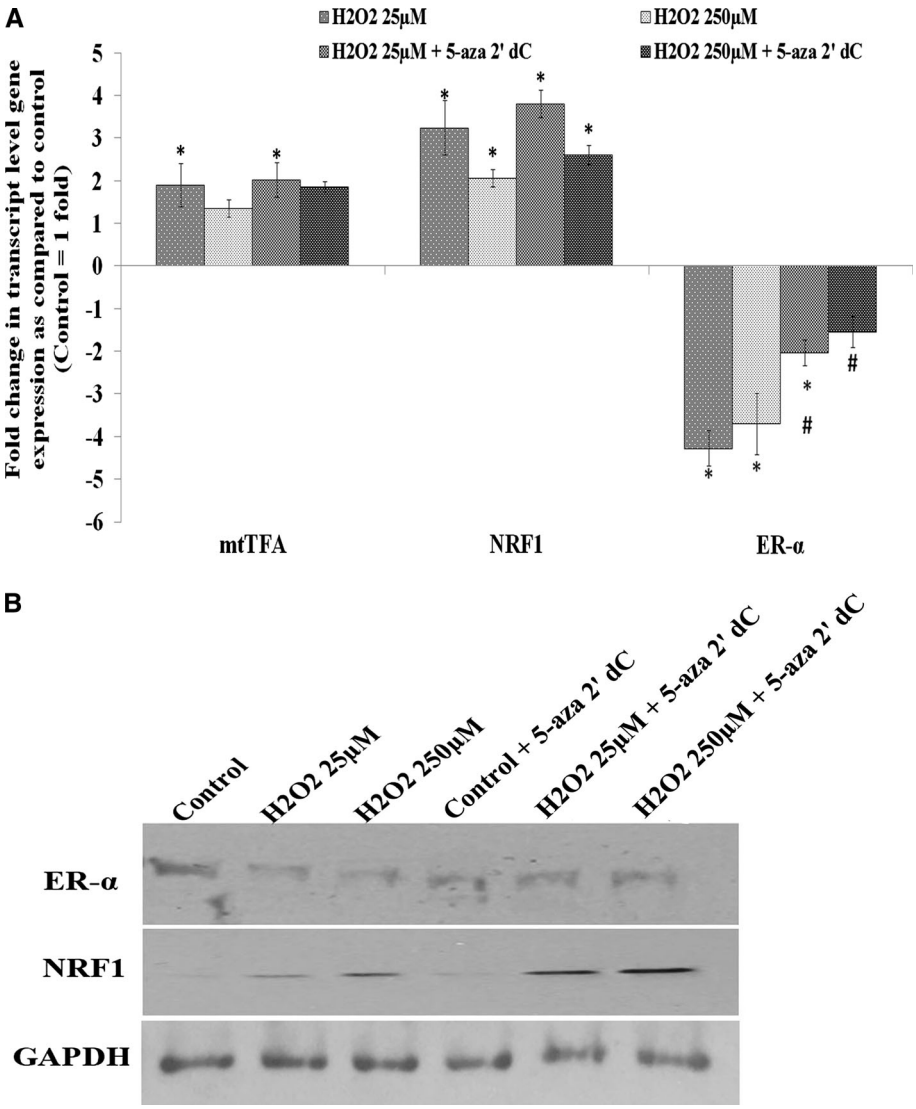

Fig. 5 Chronic oxidative stress-induced changes in expression of estrogen receptor and genes involved in mitochondrial activity. **a** Histograms representing transcript level gene expression changes evaluated by real-time PCR analysis. Total RNA isolated from MCF-7 cells chronically exposed to oxidative stress was used for one-step real-time quantitative reverse transcription PCR analysis as described in "Materials and methods" section. For each gene, C_t value was normalized to the C_t value of GAPDH and gene expression changes were calculated. The *error bars* represent the standard deviation of the mean (±SD). An *asterisk* indicates statistically significant ($p < 0.05$) change, compared to vehicle control. Symbol *hash* indicates statistically significant ($p < 0.05$) change, compared to respective group without 5-aza 2′ dC treatment. **b** Immunoblot images to show gene expression changes at protein level in MCF-7 cells exposed to chronic oxidative stress. Protein lysates from each treatment group were prepared using RIPA lysis buffer and levels of protein expression were determined by Immunoblot analysis

epigenetic regulatory genes associated with DNA methylation (*DNMT1* and *MBD4*) at protein level. In addition, levels of histone H3 modifications (Acetyl-H3K9 and Acetyl-H3K18) were also evaluated by immunoblot analysis. Results of immunoblot analysis revealed significant decrease in expression of ERα, whereas increase in expression of NRF-1 in MCF-7 cells adapted to low (25 μM) and high dose (250 μM) of chronic oxidative stress, compared to passage-matched untreated control cells (Fig. 5b). Interestingly with 5-aza 2′ dC treatment, ERα expression level in MCF-7 cells adapted to chronic oxidative stress was comparable to control cells, indicating 5-aza 2′ dC-induced reactivation of ERα expression (Fig. 5b). Further increase in expression of NRF-1 was observed in both 25 μM and 250 μM H_2O_2-exposed cells, following 5-aza 2′ dC treatment.

Significant alterations in expression of epigenetic regulatory proteins were observed in MCF-7 cells adapted to chronic oxidative stress. The levels of DNMT1 and MBD4 protein expressions were increased in both 25 and 250 μM

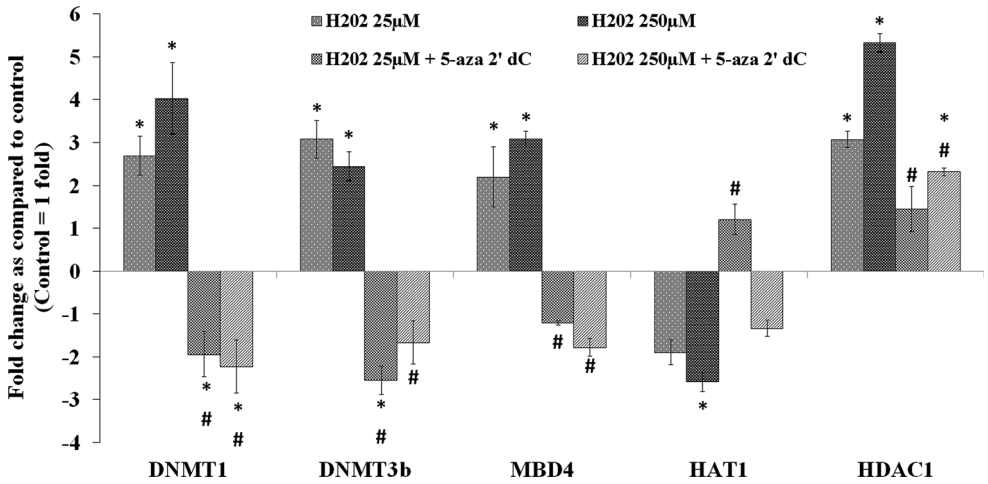

Fig. 6 Histograms representing transcript level expression changes of genes involved in epigenetic regulation. Total RNA isolated from MCF-7 cells chronically exposed to oxidative stress and one-step real-time quantitative reverse transcription PCR analysis was performed as described in "Materials and methods" section. For each gene, C_t value was normalized to the C_t value of GAPDH and gene expression changes were calculated. The *error bars* represent the standard deviation of the mean (±SD). An *asterisk* indicates statistically significant ($p < 0.05$) change, compared to vehicle control. Symbol *hash* indicates statistically significant ($p < 0.05$) change, compared to respective group without 5-aza 2′ dC treatment

H_2O_2-exposed cell. In contrast, significant decrease in DNMT1, MBD4 levels were observed following 5-aza 2′ dC treatment. Histone modifications indicative of active chromatin (acetyl-H3K9 and acetyl-H3K18) are significantly decreased in MCF-7 cells adapted to chronic oxidative stress (Fig. 7a, b). In the same cells, 5-aza 2′ dC treatment significantly increased the expression of acetyl-H3K9 levels, compared to control cells, whereas further decrease in acetyl-H3K18 levels were evident in MCF-7 cells with 5-aza 2′ dC treatment (Fig. 7a, b).

Discussion

Accumulating evidence implicates the estrogen-induced oxidative stress in initiation and progression of breast cancer [33]. However, the effects of oxidative stress on estrogen sensitivity and ER expression, the two important characteristic features that differentiate nonaggressive and aggressive forms of breast cancer, are not known. Therefore, in order to address this question, the present study was conducted, and the major finding that emerged from this study is that chronic exposure to oxidative stress causes loss of ERα expression and conversion of estrogen-dependent nonaggressive MCF-7 breast cancer cells into estrogen-independent aggressive phenotype. In addition, for the first time, our data showed that chronic oxidative stress-induced aggressive phenotype and inactivation of ERα expression in MCF-7 cells are potentially mediated by epigenetic mechanism.

Frequent progression of breast cancer into more aggressive and hormone-resistant phenotype is posing significant challenge for clinical management of continuously increasing population of breast cancer patients [2]. Multiple mechanisms have been proposed for progression of ER-positive breast cancer into more aggressive ER-negative phenotype. For example, gene mutations [34, 35], miRNA-mediated regulation [36–38], post translational modifications of ERα including phosphorylation mainly from activation of MAPK and PI3 K/Akt pathways [39], HIF-1α-mediated proteosomal degradation of ERα in hypoxic conditions [40, 41], and alterations in expression of ERα co-regulators like AP-1 and NF-kβ [42, 43] have been shown to be associated with acquisition of aggressive phenotype in breast cancer. However, the driving factors involved in promoting less-aggressive ER-positive breast cancer into more-aggressive form are not completely understood.

ROS are important mediators of growth factor receptor signaling and increased level of ROS leading to oxidative stress, and the resultant damages to cellular macromolecules are considered as important changes associated with breast cancer development [44, 45]. Oxidative stress-mediated signaling and anti-oxidative response pathways are active during breast carcinogenesis and have also been correlated with clinical prognosis and progression of breast cancer [17]. We have recently reported that exposure to relatively low-to-medium levels of chronic oxidative stress increases growth and tumorigenic potential of ER-positive MCF-7 breast cancer cells [20]. Similar finding was also recently reported in which when MCF-7 cells were treated at a low concentration of H2O2 (25 μM), the number and size of colonies were increased by more than twofold compared to controls, and these effects of H2O2 on MCF-7

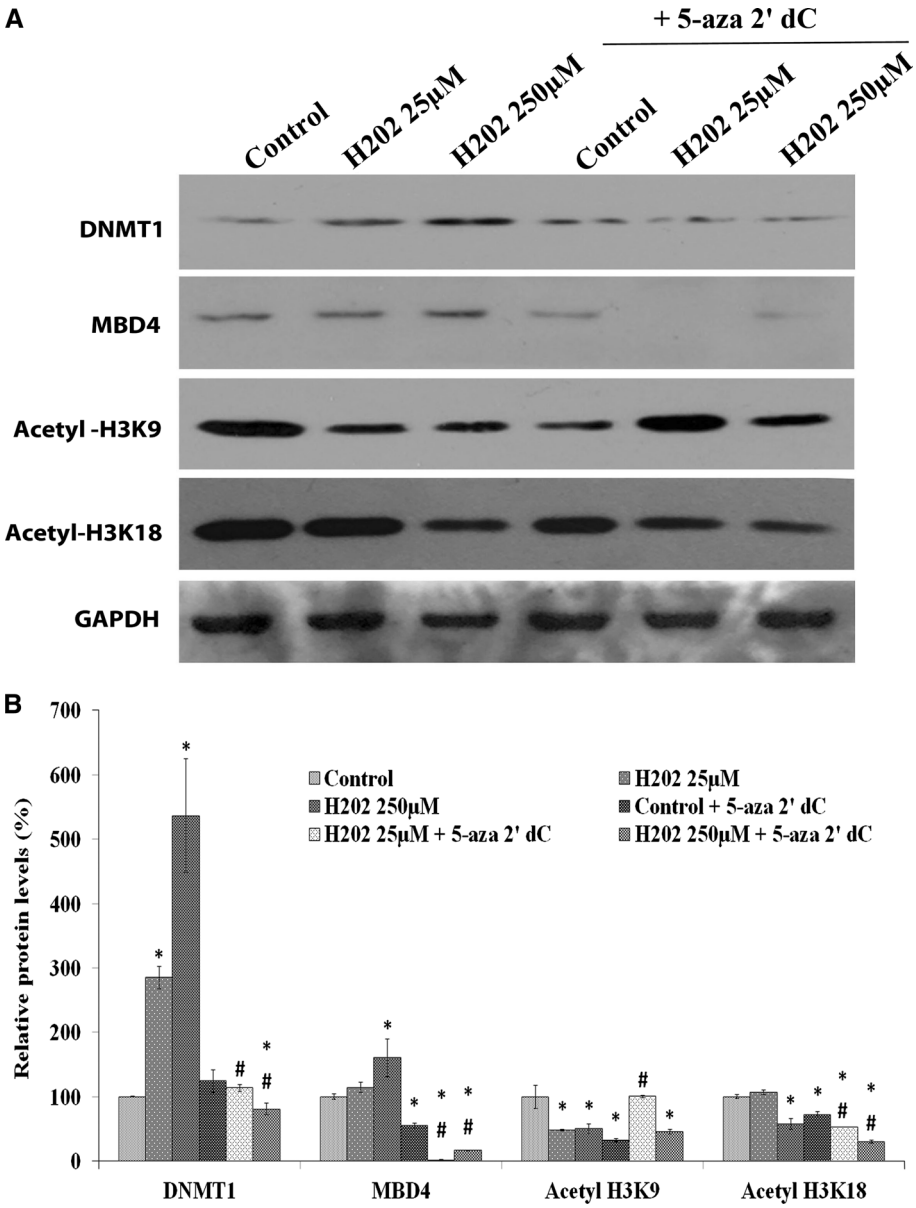

Fig. 7 Results of Immunoblot analysis showing changes in the expression of epigenetic regulatory genes at protein level. **a** Representative image of Immunoblot analysis showing changes in MCF-7 cells adapted to chronic oxidative stress with and without demethylation treatment. Protein lysates from each treatment group were prepared using RIPA lysis buffer, and levels of protein expression were determined by using specific antibody by Immunoblot analysis procedure described in "Materials and methods" section. **b** Histogram representing relative signal intensity of protein bands in different treatment groups, compared to respective control. Signal intensity of bands was measured (in triplicates) by Image J software, and each value was normalized to GAPDH signal intensity and mean fold change in signal intensity compared to respective control group were presented in an histogram. The *error bars* represent the standard deviation of the mean (±SD). An *asterisk* indicates statistically significant ($p < 0.05$) change, compared to vehicle control. Symbol *hash* indicates statistically significant ($p < 0.05$) change, compared to respective group without 5-aza 2′ dC treatment

colony were prevented by co-treatment with 500 μg/mL PEG-CAT. However, the high concentration of H2O2 (600 μM) resulted in decrease in both parameters of colonies compared to control [24]. To further understand the mechanistic basis for chronic oxidative stress-induced aggressiveness in ER-positive MCF-7 breast cancer cells and to evaluate whether these cells are still estrogen dependent for their growth, or have acquired estrogen-independent growth potential, a characteristic feature of aggressive form of breast cancer, the sensitivity of these cells to estrogen were analyzed. Interestingly, the result of this study revealed that besides the aggressive growth potential, the cells exposed to chronic oxidative stress have also become nonresponsive to 17-β estradiol-induced

growth. In addition, the estrogen antagonist tamoxifen had no effect in inhibiting the growth of these cells, thereby further supporting that growth of these cells are not dependent on estrogen. These results indicated that MCF-7 cells adapted to chronic oxidative stress are no longer regulated by estrogens/anti-estrogens, but the increased growth and aggressiveness are due to acquisition of estrogen-independent mechanisms. Growth responses to estrogen and anti-estrogen in our MCF-7 cell model (adapted to chronic oxidative stress) were similar to more aggressive and ER-negative MDA-MB-231 breast cancer cells. Significant decrease in ERα expressions both at transcript and protein levels observed in our cell model also confirms loss of estrogen dependency in MCF-7 cells during the process of adaptive response to chronic oxidative stress. ERα status has been considered as critical indicator of malignancy and aggressive behavior of breast cancer [46]. Loss of ERα expression has been reported as an important step during breast cancer progression, and resistance to endocrine therapy, leading to increased aggressiveness and poor prognosis of breast cancer patients [47, 48]. Previous studies also suggest a role of chronic oxidative stress in regulating ER status in breast cancer cells. For example, the increase in the expression of anti-oxidative response genes is concurrent with functional loss of ERα represent poor prognosis in ER-positive breast cancer cells [23]. In our study, wound healing assay results also confirmed increased migration potential of MCF-7 cells with loss of ERα expression following exposure to chronic oxidative stress. Therefore, these previous reports and the finding of this study suggest that chronic exposure to oxidative stress leads to acquisition of aggressive form in breast cancer cells through the decreased expression of ER and loss of estrogen dependency for their aggressive growth characteristic feature.

How these breast cancer cells that are adapted to chronic oxidative stress acquire aggressive growth potential without their dependency on growth hormone estrogen is another important question. Mitochondria are a major source of energy and have been shown to play an important role in growth signaling of breast cancer cells [49, 50]. Therefore, the effect of chronic oxidative stress on mitochondria was studied by analyzing the expression of mitochondrial transcription factor A (mtTFA). *mtTFA* is a nuclear-encoded gene that controls mitochondrial function, and its expression is regulated by NRF-1, a redox-sensitive transcription factor [51]. In this study, significant increases in *mtTFA* and *NRF-1* expressions were observed in MCF-7 cells after chronic exposure to oxidative stress. Similar results of aberrant mitochondrial function with increased mtTFA have been reported to be associated with increased aggressiveness and poor clinical prognosis in endometrial cancer patients [52]. Recent study also implicates the mitochondrial activity in the development of tamoxifen resistance in MCF-7 cells [53]. Hence, results of this study suggest that adaptive response and aggressive growth property of MCF-7 cells exposed to chronic oxidative stress are associated with constitutive activation of mitochondria to generate increased levels of ROS through increase in mtTFA and its transcriptional regulator NRF-1 probably to activate mitochondria-dependent redox-sensitive growth signaling pathways as an alternate to estrogen-dependent growth signaling in MCF-7 cells.

Mechanisms through which the chronic oxidative stress suppressed the ERα expression are another important question that was addressed in this study. Previous studies have proposed direct oxidative damage to alter the structure and the function of redox-sensitive zinc finger transcription factor ERα as a mechanism for its inactivation [23, 54]. The absence of significant genetic alterations (such as insertions, deletions, or point mutations) in the ERα gene evaluated in more aggressive ER-negative breast cancer cells [55] supports the potential role of epigenetic changes in regulating ERα expression. This is further supported by several reports suggesting that epigenetic changes in ERα promoter region play a major role in the loss of ERα expression and the development of hormone resistance during breast cancer progression [56–58]. Growing evidences also strongly indicate role of oxidative stress-induced epigenetic changes in breast cancer progression. Therefore, the role of epigenetic changes of DNA hypermethylation in inactivation of ERα in MCF-7 cells adapted to chronic oxidative stress was determined in this study. Significant increase in expressions of epigenetic regulatory proteins, such as DNMT1 and DNMT3b, suggested increased DNA methylation in oxidative stress-adapted MCF-7 cells. Significantly increased level of DNMT1 in ERα-negative breast cancer cells, compared to ERα-positive breast cancer cells, has been documented by previous reports [59, 60]. In addition, increase in DNMT3b expression has also been correlated with loss of ERα expression and poor prognosis in breast cancer patients [61]. Interestingly, the treatment with DNA-demethylating agent 5-aza 2′ dC resulted in the re-expression of ERα and restoration of cellular sensitivity to E2-induced proliferative response. This suggests that inactivation of ERα in MCF-7 cells adapted to chronic oxidative stress was due to DNA hypermethylation. Similar results showing role of DNA hypermethylation in inactivation of ERα expression have been reported earlier. For example, DNA hypermethylation-mediated silencing of ERα expression was observed in 41 % of breast cancer patients and was also correlated with aggressiveness and size of tumor [58, 62]. Increases in the number of hypermethylated CpG islands (at global genome wide level) with progression of differentiated cancer into undifferentiated aggressive form were

reported in breast cancer patients [63]. In ER-positive breast cancer cells, both proximal and distal promoter regions of ERα remain largely unmethylated, whereas significant increase in methylation in these promoter regions has been reported in ER-negative breast cancer cells [16, 64, 65]. In addition, ERα restoration in ER-negative breast cancer cells with siRNA-mediated silencing of enzymes involved in methyl transfer (*DNMT1*) and by chemicals which inhibit DNA methylation (5-aza 2′ dC) further strengthens the role of epigenetic mechanism [27].

To further gain an insight into the epigenetic mechanism for oxidative stress-induced aggressive growth characteristics and loss of estrogen sensitivity in MCF-7 cells, additional epigenetic regulatory genes and histone modifications were also analyzed. Increased methylation of gene promoter normally results in the inhibition of gene expression by blocking binding of transcription factors or through recruitment of proteins which specifically bind to methylated DNA like MBD proteins, which recruit HDACs and other proteins to induce repressive chromatin [66]. Significant increase in MBD4, a methyl-binding protein, was observed in our MCF-7 cell model exposed to chronic oxidative stress. Previous reports also documented increased localization of MBD proteins in ERα promoter region of ER-negative MDA-MB 231 breast cancer cells, compared to MCF-7 cells [67]. In addition, knock down of MBD4 protein results in reprogramming of hypermethylated DNA regions to level of methylation comparable to basal level and inhibition of metastasis in MCF-7 cells [68]. Hence, the results of our study in oxidative stress model were in accordance with previous reports with respect to role of MBD proteins in epigenetic repression of ERα.

In addition, interaction between DNA methylation and histone modifications mainly acetylation/deacetylation are also important for transcriptional silencing of genes. Results of RT-PCR analysis in this study showed significant increases in both DNA methyl transferases (*DNMT1*) and histone deacetylases (*HDAC1*) expressions in MCF-7 cells adapted to oxidative stress. Interaction of DNMT1 with HDAC proteins is known to induce repressive chromatin formation [69, 70]. In breast cancer, roles of DNMTs and HDAC proteins in regulating ERα expression have been documented by multiple researchers through re-expression of ERα by using DNMT and HDAC inhibitors [71, 72]. HDAC1 induction in MCF-7 cells is associated with increased proliferation and loss of ERα expression [73]. Hence, the results of our study support the role of interaction between chronic oxidative stress-induced DNA methylation and histone acetylation in regulating ERα expression. Both acetyl-H3K9 and acetyl-H3K18 are markers for transcriptionally active chromatin [46]. The results of immunoblot analysis in this study showed significant decreases in acetyl-H3K9 and acetyl-H3K18 levels in MCF-7 cells exposed to low and high levels of chronic oxidative stress, suggesting thereby that the observed decrease in ERα expression could potentially also be mediated by repressive chromatin due to decreased acetylation and/or increased deacetylation of histone tail. Decreased expression of HAT1 and increased expression of HDAC1 enzymes at transcript in both low and high doses of H_2O_2-exposed MCF-7 cells further support the role of histone modifications in chronic oxidative stress-induced loss of ERα expression. Following 5-aza 2′ dC treatment, significant decrease was observed only in acetyl-H3K9 modification. However, in the level of acetyl-H3K18, changes were increased in low dose MCF-7 cells after 5-aza 2′ dC treatment, whereas no significant change was observed in high-dose cells. Similar to results of our model, significant decrease in acetyl-H3K9 (active) and increase in trimethyl-H3K9 (inactive) were reported at ERα promoter region in ERα-negative MDA-MB-231 breast cancer cells, when compared to MCF-7 cells [46].

In conclusion, the data of this study suggest that MCF-7 cells adapted to chronic oxidative stress acquire estrogen independence, aggressive growth potential, and resistance to endocrine therapy, potentially through epigenetic silencing of ERα expression. Results of our study will be useful to understand molecular mechanisms associated with progression of breast cancer into more aggressive subtype and will be helpful to further evaluate usefulness of antioxidants and/or epigenetic modifying drugs to inhibit progression of breast cancer into more aggressive form and to improve the clinical outcome in breast cancer patients.

Compliance with ethical standards

Conflict of interest The authors hereby disclose that there are no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work, and therefore there is no conflict of interest.

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