Thioredoxin and thioredoxin reductase influence estrogen receptor α-mediated gene expression in human breast cancer cells

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Abstract

Accumulation of reactive oxygen species (ROS) in cells damages resident proteins, lipids, and DNA. In order to overcome the oxidative stress that occurs with ROS accumulation, cells must balance free radical production with an increase in the level of antioxidant enzymes that convert free radicals to less harmful species. We identified two antioxidant enzymes, thioredoxin (Trx) and Trx reductase (TrxR), in a complex associated with the DNA-bound estrogen receptor α (ERα). Western analysis and immunocytochemistry were used to demonstrate that Trx and TrxR are expressed in the cytoplasm and in the nuclei of MCF-7 human breast cancer cells. More importantly, endogenously expressed ERα, Trx, and TrxR interact and ERα and TrxR associate with the native, estrogen-responsive pS2 and progesterone receptor genes in MCF-7 cells. RNA interference assays demonstrated that Trx and TrxR differentially influence estrogen-responsive gene expression and that together, 17β-estradiol, Trx, and TrxR alter hydrogen peroxide (H2O2) levels in MCF-7 cells. Our findings suggest that Trx and TrxR are multifunctional proteins that, in addition to modulating H2O2 levels and transcription factor activity, aid ERα in regulating the expression of estrogen-responsive genes in target cells.

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Introduction

Eukaryotic cells consume oxygen and produce reactive oxygen species (ROS) as by-products of normal cellular metabolism (Powell et al. 2005). ROS include a number of chemically reactive oxygen derivatives including superoxide and hydrogen peroxide (H2O2), which are less reactive, and hydroxyl radical, which is highly reactive. The initial product of oxygen metabolism, superoxide, is dismutated to H2O2 in cells by superoxide dismutase (SOD). The H2O2 is then converted to H2O and O2 by catalase, glutathione peroxidase, and peroxiredoxins, which include thioredoxin (Trx) peroxidases (Beckman et al. 1990, Webster et al. 2001, Yoshida et al. 2003, Smart et al. 2004, Hashemy et al. 2006).

ROS are needed to serve as molecular messengers in cell-signaling pathways and in the immune system to target pathogens (Lehnert & Iyer 2002, Feinendegen 2005, Goldstein et al. 2005). At low concentrations, superoxide and H2O2 are effective stimulators of cell growth (Burdon 1995). However, if H2O2 is not effectively eliminated, hydroxyl radicals can accumulate and damage proteins, lipids, and DNA (Halliwell & Gutteridge 1985, Storz et al. 1990). Increased ROS accumulation has also been linked to tumorigenesis and age-related diseases (Kirkwood & Austad 2000, Toussaint et al. 2002) as well as decreased cell survival (Salganik 2001). Thus, oxygen radicals have beneficial as well as detrimental effects.

In order to avoid ROS accumulation and its damaging effects, cells express a battery of oxidative stress response proteins that dissipate oxygen radicals (Halliwell & Gutteridge 1985, Storz et al. 1990). Trx is an oxidative stress response protein that activates i) transcription factors in order to alter gene expression and ii) peroxiredoxins, so that cellular H2O2 can be diminished (Webster et al. 2001, Arner & Holmgren 2006). Like the proteins that it reduces, Trx itself must be reduced in order to activate other proteins. Trx reductase (TrxR) utilizes NADPH to reduce and activate Trx as well as other proteins (Mustacich & Powis 2000). By maintaining protein thiols in the reduced state, Trx and TrxR help to maintain a reduced cellular environment and active transcription factors (Holmgren 1979, 1985, Das et al. 1997).

Zinc finger proteins are particularly susceptible to oxidation. Oxidation of zinc finger proteins diminishes the ability of these proteins to interact with their target DNA sequences and ultimately alters gene expression.
Oxidation of two nuclear receptor superfamily members, the glucocorticoid receptor and estrogen receptor α (ERα), diminishes the ability of these proteins to bind to DNA (Makino et al. 1996, Hayashi et al. 1997).

ERα binds to hormone, dimerizes, interacts directly with its receptor elements in DNA, estrogen-responsive elements (EREs), and recruits coregulatory proteins that influence estrogen-responsive gene expression. Because we had previously shown that three other oxidative stress proteins, SOD1, protein disulfide isomerase (PDI), and apurinic/apyrimidinic endonuclease 1 or redox factor-1 (Ape1/Ref-1) influence ERα-mediated gene expression (Schultz-Norton et al. 2006, Rao et al. 2008, Curtis et al. 2009), we were intrigued by the identification of Trx and TrxR in a complex of proteins associated with the DNA-bound ERα (Schultz-Norton et al. 2008, 2009) and were interested in determining whether Trx and TrxR might also influence ERα-mediated gene expression. We now show that Trx and TrxR alter estrogen-responsive gene expression and that together, 17β-estradiol (E2), Trx, and TrxR modulate H2O2 levels in MCF-7 human breast cancer cells.

Materials and methods

Cell culture

MCF-7 human breast cancer cells, which express ERα, were maintained in phenol red-containing minimum essential medium (MEM, Invitrogen) with 1× non-essential amino acids (NEAA, Invitrogen), 20 mM HEPES, and antibiotics (penicillin–streptomycin and gentamicin) with 5% calf serum. Cells were switched to phenol red-free MEM with 5% charcoal/dextran-treated calf serum (CDCS, Eckert & Katzenellenbogen 1982), NEAA, and antibiotics for 1–3 days before experiments were initiated. MDA-MB-231 human breast cancer cells, which do not express ERα, were maintained in Leibovitz’s L-15 medium (Invitrogen) with the same additives as used for MCF-7 cells. U2 osteosarcoma (U2OS) cells were maintained in MEM with 15% heat-inactivated FCS and with the same additives as used for MCF-7 cells. HeLa cervical cancer cells were maintained in DMEM /Nutrient Mixture F-12 Ham with 5% heat-inactivated FCS and penicillin–streptomycin.

Isolation and identification of Trx and TrxR

Trx and TrxR were isolated as proteins associated with the ERE-bound ERα using agarose gel shift assays and identified using mass spectrometry analysis as previously described (Schultz-Norton et al. 2008, 2009). Three peptides that contained amino acid sequences unique to Trx1 (TAFAQEALDAAGDKLVVDF-SATWCGBPCK, PFFHSLSEK, and EKLEATINELV) and 11 peptides that contained amino acid sequence identical to TrxR1 (VMVLDFTPTPLGTRWGTLGGT-VNVGCIPKLMHQALLGQALQDSR, MIAEAVQHIG SLNWGYR, KVVENNAVQFIQPFR, FLIATGERPR, IGEHMEEHGIK, QFVIPKVEEQATGPR, VQAQSTN SEEIIIEGNYTMLAIQR, IPYTDREQTNVPIYAIAGDLEDKVFELTPVAIQAGR, FGEENIEVHSYFPLEW TIPS, VVGFHYLPGNAEGTVTQGFAAALK, and QLDESTIGHPVCAEVFTITLSVTK) were identified. Together, these peptides account for 45-7 and 49-7% of the total Trx and TrxR amino acid sequences respectively.

Western-blot experiments

Nuclear extracts from human breast (MCF-7 and MDA-MB-231), bone (U2OS), and cervical (HeLa) cancer cells were prepared as described (Wood et al. 2001). Ten micrograms of nuclear extract were fractionated on 10–18% SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane, which was probed with a Trx-, TrxR-, ERα- (sc-20146, sc-28321, sc-8002 respectively, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), PR-A and PR-B (RM-9102-S1, Lab Vision, Fremont, CA, USA), or GAPDH- (TAB1001, Open Biosystems, Huntsville, AL, USA) specific antibody and a HRP-conjugated secondary antibody. Proteins were detected by the SuperSignal West Femto Maximum Sensitivity Substrate chemiluminescent system (Pierce, Rockford, IL, USA).

Immunocytochemistry

MCF-7 cells were plated onto coverslips in six-well plates containing phenol red-free MEM with NEAA, antibiotics, and 5% CDCS. Three wells were treated with ethanol vehicle or with 10 nM E2. After 24 h, cells were washed with PBS, fixed in PBS with 4% formaldehyde for 10 min, washed with PBS, permeabilized with PBS containing 0.1% Triton X-100 for 20 min, and washed with PBS containing 0.1% Tween 20 (PBST). Samples were blocked with PBST containing 2% BSA and 2% fetal bovine serum for 30 min, incubated with a Trx- or TrxR-specific antibody (sc-20146 and sc-28321 respectively, Santa Cruz Biotechnologies) for 1 h in a humidified chamber, washed with PBST, incubated with donkey anti-rabbit biotin-SP-conjugated antibody (Trx, 711-066-152) or donkey anti-mouse biotin-SP-conjugated antibody (TrxR, 715-066-150, Jackson ImmunoResearch, West Grove, PA, USA) for 30 min, washed with PBST, incubated with DyLight 549-conjugated Streptavidin (016-500-084, Jackson ImmunoResearch, West Grove, PA, USA) for 30 min and washed in PBS. Primary antibodies were omitted in control slides and
run in parallel to demonstrate the specificity of the Trx and TrxR antibodies. Samples were mounted with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) and visualized with a 40X objective using a Leica DM4000 B confocal microscope (Leica Microsystems, Inc., Bannockburn, IL, USA) with the Leica TCS SPE system and Application Suite Advanced Fluorescence software. Three fields were examined in three independent experiments so that nine fields were examined for each treatment.

**Immunoprecipitation assays**

MCF-7 cells were treated with ethanol or 10 nM E2 for 0-75 h, washed with PBS, harvested in 20 mM Tris pH 7-4, 10 mM EDTA, 100 mM NaCl, 0.5% NP-40, 1 mM Na3VO4, 50 mM NaF, and 1× protease inhibitor cocktail (PIC, Sigma), and then pelleted at 20 800 g at 4 °C for 10 min. The protein concentration of each supernatant was determined using the Bio-Rad protein assay (Bio-Rad) with BSA as a standard. One microgram of Trx- or TrxR-specific antibody (sc-18215 or sc-31057 respectively, Santa Cruz Biotechnologies) or a control antibody directed against fluorescein (Immuno logical Resource Center, University of Illinois, Urbana, IL, USA) was incubated with 500 µg of extract overnight at 4 °C with rotation, incubated with 60 µl of a 50% Protein G Sepharose slurry for 1 h (GE Healthcare, Piscataway, NJ, USA), and centrifuged at 960 g at 4 °C for 2 min. Samples were washed thrice with 20 mM Tris pH 7-4, 10 mM EDTA, 100 mM NaCl, 0-1% NP-40, 1 mM Na3VO4, 50 mM NaF, and 1× PIC before fractionation on SDS-polyacrylamide gels and western analysis with an ER-z-specific antibody (sc-543, Santa Cruz Biotechnologies).

**Chromatin immunoprecipitation assays**

MCF-7 cells were treated with ethanol or 10 nM E2 for 0-75 or 24 h and incubated with 1% formaldehyde. Cells were processed essentially as described (Curtis et al. 2007). Antibodies directed against ERz or TrxR (sc-8002 and sc-31057 respectively, Santa Cruz Biotechnologies) were used for chromatin immunoprecipitation (ChiP). Primers specific to the ERE-containing region of the pS2 gene or two regions of the progesterone receptor (PR) gene (JL Boney-Montoya, YS Ziegler, CD Curtis, JA Montoya & AM Nardulli, unpublished observations), located 205 kb (PR205) or 221 kb (PR221) upstream of the PR-B transcription start site, were used for real-time PCR with iQ SYBR Green Supermix (Bio-Rad) and the iCycler PCR thermocycler. Standard curves were generated using 1000, 5000, 10 000, 50 000, and 100 000 copies of each gene for each primer set in each experiment and run in parallel.

**RNA interference experiments**

MCF-7 cells were transferred to phenol red-free MEM with NEAA and antibiotics with 5% CDCS 1 day prior to plating. Cells were seeded in 12-well plates 24 h before transfection with siLentFect (Bio-Rad) and transferrin (Sigma). We combined 50 pmol small interfering RNA (siRNA) directed against TrxR (117158 or 111300), TrxR (111302 or 41855), or control Renilla luciferase (4630, Ambion, Austin, TX, USA) with 500 µl phenol red-free medium and incubated this with the cells for 24 h. Cells were then incubated in phenol red-free medium containing ethanol or 10 nM E2 for 24 h. For protein analysis, cells were harvested with TNE (10 mM Tris, pH 7.5, 150 mM NaCl, and 1 mM EDTA), lysed in lysis buffer (20 mM Tris pH 8, 1 mM EDTA, 200 mM NaCl, and 0-2% NP-40), and subjected to western blot analysis with a Trx-, TrxR- (sc-20146 and sc-28321, Santa Cruz Biotechnologies), PR-A- and PR-B- (RM-9102-S1, Lab Vision), or GAPDH- (TAB1001, Open Biosystems) specific antibody. For RNA analysis, cells were resuspended in TRIzol (Invitrogen) and processed according to the manufacturer’s instructions. cDNA was prepared using the Reverse Transcription System (Promega), and real-time PCR was performed using iQ SYBR Green Supermix and the iCycler PCR thermocycler (Bio-Rad) with primers specific to Trx (5′-CTTCTTTTCTCCCTCT TG-3′ and 5′-GCA TTTGACCTTACA CTCGTG-3′), TrxR (5′-TGGAAATTTG GTGCTTGTG-3′ and 5′-ATATCTTTGAC-GAATCGT-3′), pS2, PR, cyclin G2, cyclin D1, Bcl2, and 36B4 (Curtis et al. 2007, Creekmore et al. 2008). Standard curves were derived using cDNA equivalents of 0-2, 0-2, 2, and 20 ng of RNA and were run in duplicate with each primer set in each experiment.

**H2O2 quantitation**

MCF-7 cells were treated with control, Trx or TrxR siRNA, and ethanol or E2 as described for siRNA experiments. Cells were harvested and centrifuged at 960 g for 5 min at 4 °C. The pelleted cells were resuspended in lysis buffer and spun at 20 800 g for 5 min at 4 °C. The supernatants were transferred to a 96-well plate and Amplex Red (Invitrogen), which interacts with H2O2 to produce the red fluorescent oxidation product resorufin, was used to determine the level of endogenous H2O2 (Invitrogen). To derive a standard curve, duplicate samples, which included 250, 500, 750, and 1000 nM of H2O2, were run in parallel. All samples were analyzed with a fluorescence plate reader using 544 nm for excitation and 590 nm for fluorescence detection. Protein concentrations were determined using the Bio-Rad protein assay
254  A K RAO and others • Trx and TrxR influence estrogen responsiveness

expression of Trx, but not
Interestingly, when MCF-7 cells were treated with 10 nM E2 for 24 h, the expression of Trx, but not TrxR, was enhanced (Fig. 1B). A GAPDH-specific antibody was used to demonstrate that similar amounts of protein were loaded in each lane.

ERα resides in the nuclei of MCF-7 cells (Schultz-Norton et al. 2006). Although Trx and TrxR have been localized in the nuclei of cultured cells, they have more often been described as cytoplasmic proteins (Arner & Holmgren 2000, Nordberg & Arner 2001, Yoshida et al. 2003). To determine whether Trx and TrxR were present in the nuclear compartment of MCF-7 cells where they might be able to interact with ERα and influence gene expression, immunocytochemistry was performed. As seen in Fig. 1C, Trx and TrxR were expressed in the cytoplasm, but were also present in the nuclei of MCF-7 cells. Exposure of MCF-7 cells to E2 for 24 h dramatically increased Trx expression. The increase in Trx expression might be expected since it has been reported that ERα is associated with the Trx gene in MCF-7 cells (Carroll et al. 2005). No changes were observed in TrxR expression or the localization of Trx and TrxR with hormone treatment. Thus, although previous studies have reported the ability of ionizing radiation, nitric oxide, or oxidative stress to induce translocation of Trx to the nucleus (Hirota et al. 1999, Wei et al. 2000, Arai et al. 2006), E2 did not alter the localization of Trx or TrxR in MCF-7 cells. These findings are consistent with those reported in the Human Protein Atlas (www.proteinatlas.org).

Endogenously expressed Trx, TrxR, and ERα interact

Because Trx and TrxR were originally isolated in a large complex associated with the DNA-bound ERα using HeLa nuclear extracts (Schultz-Norton et al. 2008, 2009), we determined whether endogenously expressed Trx, TrxR, and ERα from MCF-7 cells could interact. Trx- and TrxR-specific antibodies were used to immunoprecipitate the proteins from MCF-7 extracts, and then western analysis was performed with an ERα-specific antibody. ERα was immunoprecipitated with Trx- (Fig. 2A, lanes 5 and 6) and TrxR- (Fig. 2B, lanes 5 and 6) specific antibodies. In contrast, a control antibody directed against fluorescein was unable to immunoprecipitate ERα regardless of whether cells had or had not been exposed to E2 (lanes 3 and 4). These studies demonstrate that endogenously expressed Trx and TrxR associate with ERα in the absence and in the presence of hormone.

Statistical analysis

SAS 9-1 Basic Statistics (SAS Institute, Cary, NC, USA) was used for statistical analysis. One-way ANOVA was used to determine whether there were differences between control and experimental groups. A P-value ≤0.05 was considered to be statistically significant.

Results

Trx and TrxR are present in cytoplasmic and nuclear compartments

We originally identified Trx and TrxR as proteins associated with the ERE-bound ERα (Schultz-Norton et al. 2008, 2009). Although HeLa nuclear extracts had been utilized in these initial experiments, we examined the expression of these two proteins in various cultured cell lines that have been used to study estrogen responsiveness (Druege et al. 1986, Green et al. 1986, Greene et al. 1986, Katzenellenbogen et al. 1987). Western blot analysis with Trx- and TrxR-specific antibodies demonstrated that both proteins are expressed in ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) human breast cancer cells, U2OS, and, as expected, HeLa cervical cancer cells (Fig. 1A). Interestingly, when MCF-7 cells were treated with 10 nM E2 for 24 h, the expression of Trx, but not TrxR, was enhanced (Fig. 1B). A GAPDH-specific antibody was used to demonstrate that similar amounts of protein were loaded in each lane.

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TrxR associates with endogenous estrogen-responsive genes

The interaction of endogenously expressed ERα, Trx, and TrxR (Fig. 2) and the association of Trx and TrxR with the DNA-bound ERα in vitro (Schultz-Norton et al. 2008, 2009) led us to investigate whether these proteins associate with native estrogen-responsive genes in MCF-7 cells. Using ChIP assays, we previously demonstrated that ERα and other coregulatory proteins associate with an ERE-containing region of the pS2 gene and with two regions of the PR gene located 205 kb (PR205) and 221 kb (PR221) upstream of the PR-B transcription start site (JL Boney-Montoya, YS Ziegler, CD Curtis, JA Montoya & AM Nardulli, unpublished observations, Schultz-Norton et al. 2006, 2007, Curtis et al. 2007, Creekmore et al. 2008, Rao et al. 2008). PR205 and PR221 contain one and two imperfect EREs respectively.

Significantly more ERα (Fig. 3A) and TrxR (Fig. 3B) were associated with the ERE-containing regions of the pS2 and PR genes in the presence than in the absence of E2, suggesting that TrxR may influence estrogen responsiveness of these genes by associating with the DNA-bound ERα in native chromatin. However, we were unable to detect any significant change in the association of Trx with the pS2 and PR genes in the absence and in the presence of E2 using three different Trx-specific antibodies (data not shown). This could result from the inability of the Trx-specific antibodies to effectively immunoprecipitate Trx (as suggested in Fig. 2A), a transient association of Trx with these gene regions, and/or the relatively small size of Trx (12 kDa), which could make it less accessible to antibody and more susceptible to epitope masking. No changes were observed in the association of ERα or TrxR with the internal control gene, 36B4, which was used for normalization of each sample.

TrxR and TrxR influence estrogen responsiveness

To determine more directly whether Trx and TrxR influence the expression of endogenous, estrogen-responsive genes, RNA interference assays were performed to individually knock down Trx and TrxR expression. MCF-7 cells were transfected with siRNA directed against endogenously expressed Trx or TrxR mRNA. In addition, a control siRNA directed against Renilla luciferase, which is not expressed in these cells, was utilized. Trx and TrxR siRNA successfully reduced the protein and mRNA levels of Trx (Fig. 4) and TrxR (Fig. 5) respectively. When control siRNA was used, pS2, PR, cyclin D1, and Bcl2 mRNA and PR protein levels were increased and cyclin G2 mRNA levels were decreased in the presence of E2 (Figs 4 and 5). These findings are consistent with earlier studies from our laboratory and others (Westley & May 1987, Nardulli et al. 1988, Altucci et al. 1996, Kim et al. 2000, Stossi et al. 2006, Curtis et al. 2007, Creekmore et al. 2008, Rao et al. 2008).
While the E\textsubscript{2}-induced increase in pS2 mRNA expression was reduced when Trx siRNA was included, PR mRNA and protein levels were further enhanced (Fig. 4). The E\textsubscript{2}-induced repression in cyclin G2 was enhanced, resulting in further reduction in cyclin G2 mRNA expression. In contrast, cyclin D1, Bcl2, and ER\textalpha\textsubscript{a} mRNA levels were unaltered when Trx expression was reduced. The internal control gene, 36B4, which contains no apparent ER\textalpha\textsubscript{a}-binding sites, was unaffected by E\textsubscript{2} or the Trx siRNA (Fig. 4B).

When TrxR was knocked down, pS2 and cyclin D1 mRNA levels decreased and PR and Bcl2 mRNA levels as well as PR protein increased in the presence of hormone (Fig. 5). Cyclin G2 mRNA levels were not significantly altered when TrxR expression was reduced and ER\textalpha\textsubscript{a} mRNA levels were decreased in the absence, but not in the presence of E\textsubscript{2}. Again, 36B4 mRNA levels were unaffected by E\textsubscript{2} or TrxR siRNA (Fig. 5B). The Trx and TrxR siRNAs were protein specific. Decreasing the level of one protein did not significantly affect the expression of the other (data not shown). Furthermore, two different Trx and TrxR siRNAs produced similar effects on estrogen-responsive gene expression. Taken together, these studies indicate that Trx and TrxR have gene-specific, rather than global effects, on estrogen-responsive gene expression.

Figure 4 Knocking down Trx influences estrogen responsiveness. MCF-7 cells were transfected with 50 pmol control or Trx siRNA, treated with ethanol (−E\textsubscript{2} and light gray bars) or 10 nM E\textsubscript{2} (+E\textsubscript{2} and black bars) for 24 h, and processed for protein or mRNA analysis. (A) Proteins were subjected to western analysis with an antibody that recognizes Trx, PR-A and PR-B, or GAPDH. (B) RNA was isolated and cDNA was synthesized for quantitative RT-PCR analysis with primers specific to Trx, pS2, PR, cyclin G2, cyclin D1, Bcl2, ER\textalpha\textsubscript{a}, and 36B4 (internal control) mRNA sequences. Data from three independent experiments, which had been performed in triplicate, were combined and are presented as the mean ± S.E.M. ANOVA was used to detect significant differences in mRNA levels in response to E\textsubscript{2} (**P ≤ 0.05) or in response to Trx siRNA (##P ≤ 0.05).

Figure 5 Knocking down TrxR influences estrogen responsiveness. MCF-7 cells were transfected with 50 pmol control or TrxR siRNA, treated with ethanol (−E\textsubscript{2} and light gray bars) or 10 nM E\textsubscript{2} (+E\textsubscript{2} and black bars) for 24 h, and processed for protein or mRNA analysis. (A) Proteins were subjected to western analysis with an antibody that recognizes TrxR, PR-A and PR-B, or GAPDH. (B) RNA was isolated and cDNA was synthesized for quantitative RT-PCR analysis with primers specific to TrxR, pS2, PR, cyclin G2, cyclin D1, Bcl2, ER\textalpha\textsubscript{a}, and 36B4 (internal control) mRNA sequences. Data from three independent experiments, which had been performed in triplicate, were combined and are presented as the mean ± S.E.M. ANOVA was used to detect significant differences in mRNA levels in response to E\textsubscript{2} (**P ≤ 0.05) or in response to TrxR siRNA (##P ≤ 0.05).
Trx and TrxR alter H$_2$O$_2$ levels in MCF-7 cells

It is well established that Trx influences ROS distribution by activating antioxidant enzymes that convert H$_2$O$_2$ to H$_2$O (Tam et al. 2003, Arner & Holmgren 2006). Since E$_2$ increases Trx expression (Figs 1 and 4), we hypothesized that E$_2$ treatment might affect H$_2$O$_2$ levels in MCF-7 cells. Furthermore, since TrxR is required to activate Trx, it seemed possible that TrxR might also play a role in regulating H$_2$O$_2$ levels. To test these hypotheses, MCF-7 cells that were exposed to control, Trx, or TrxR siRNA in the absence and in the presence of E$_2$ and H$_2$O$_2$ levels were measured. When control siRNA was used, H$_2$O$_2$ levels were higher in the absence than in the presence of E$_2$ (Fig. 6). This decreased level in H$_2$O$_2$ after treatment of MCF-7 cells with E$_2$ for 24 h most likely results from the dissipation of ROS by the E$_2$-mediated increase in antioxidant proteins (Mobley & Brueggemeier 2004, Rao et al. 2008).

When Trx or TrxR expression was knocked down and cells were treated with ethanol, H$_2$O$_2$ levels were significantly reduced. However, when cells were treated with E$_2$ and Trx or TrxR siRNA, H$_2$O$_2$ levels were enhanced. These studies demonstrate that individually and collectively Trx, TrxR, and E$_2$ alter H$_2$O$_2$ levels in MCF-7 cells.

Discussion

Previous studies have shown that Trx and TrxR help to dissipate ROS, maintain a reduced intracellular environment, and protect cellular macromolecules from oxidative damage (Holmgren 1985, Holmgren & Bjornstede 1995, Osborne et al. 2001, Lincoln et al. 2003, Smart et al. 2004). We now demonstrate that endogenously expressed Trx and TrxR interact with ERz and alter estrogen-responsive gene expression and that together, Trx, TrxR, and E$_2$ influence H$_2$O$_2$ levels in MCF-7 human breast cancer cells.

Effect of Trx and TrxR on H$_2$O$_2$ levels

Although a previous study reported that ROS levels increase when MCF-7 cells are treated with 100 μM E$_2$ for 15 min (Felty et al. 2005), we, in fact, observed an E$_2$-dependent reduction in H$_2$O$_2$ levels when MCF-7 cells had been exposed to 10 nM E$_2$ for 24 h. While the 1000-fold difference in E$_2$ concentrations used in these two studies might account for some of the difference observed, we believe that the diminished H$_2$O$_2$ levels observed after 24 h of E$_2$ treatment are primarily due to the increased expression of oxidative stress proteins such as SOD1 (Rao et al. 2008) and Trx (Figs 1 and 4), increases that would not be observed after a 15 min exposure to E$_2$. In addition, although an earlier study monitored H$_2$O$_2$ levels after the addition of exogenous H$_2$O$_2$ to the culture media (Mobley & Brueggemeier 2004), these experiments are distinctly different from our studies in which cells were exposed to vehicle or hormone, and endogenous production of H$_2$O$_2$ was measured. Overall, our findings support the idea that E$_2$ plays an important role in regulating H$_2$O$_2$ levels in MCF-7 cells by modulating the expression of oxidative stress proteins.

Effects of Trx and TrxR on estrogen-responsive gene expression

The capacity of Trx to reduce peroxiredoxins, which convert H$_2$O$_2$ to H$_2$O (Arner & Holmgren 2006), helps to maintain a reduced intracellular environment and ensure that transcription factors are in a reduced, active state. Thus, Trx along with its activator, TrxR, help in the overall maintenance of transcription factor-binding activity.

The zinc fingers of ERz provide the specificity required for recognizing and interacting with ERE-containing DNA, but are sensitive to oxidative stress (Webster et al. 2001). Although oxidation of ERz by ROS or the oxidizing agent diamide inhibits the ability of the receptor to interact with ERE-containing DNA, its DNA-binding capacity can be restored by the reducing agent dithiothreitol or Trx (Hayashi et al. 1997). The ability of Trx and TrxR to help maintain ERz structure and function is evident in the altered estrogen-responsive gene expression when either protein is knocked down (Figs 4 and 5). Trx also plays an active role in maintaining the DNA-binding activity of other transcription factors including NF-kB, cAMP response element binding protein, p53, Sp1, AP-1 proteins, and the glucocorticoid receptor (Matthews et al. 1992, 1997, Webster et al. 2001).

Figure 6 Trx, TrxR, and E$_2$ modulate H$_2$O$_2$ levels. MCF-7 cells were transfected with 50 pmol control, Trx, or TrxR siRNA and treated with ethanol (−E$_2$) or 10 nM E$_2$ (+E$_2$) for 24 h. Cell extracts were prepared and incubated with Amplex Red to detect the levels of H$_2$O$_2$. Data from three independent experiments were combined and are expressed as the mean±S.E.M. ANOVA was used to detect significant differences in the levels of H$_2$O$_2$ in the presence of E$_2$ (*P≤0.05) or in response to Trx or TrxR siRNA (#P≤0.05).
Although we were unable to detect a difference in the association of Trx with the pS2 and PR genes in the absence and in the presence of hormone, the isolation of Trx in a complex with the DNA-bound ERα (Schultz-Norton et al. 2008, 2009), the immunoprecipitation of ERα with a Trx-specific antibody in the absence and in the presence of hormone (Fig. 2A), and the ability of Trx to reduce ERα and enhance its binding to DNA (Hayashi et al. 1997) suggest that Trx associates with ERα at target genes. The E2-induced increase in the association of TrxR with the pS2 and PR genes in the presence of hormone (Fig. 3B) could help to ensure that any Trx associated with these gene regions is active and capable of reducing ERα and its associated coregulatory proteins and influencing transcription. Thus, TrxR, through its modulation of Trx activity, may play a role in reducing proteins and maintaining a reduced environment (Arner & Holmgren 2000, Nordberg & Arner 2001). In addition, TrxR has been referred to as a ‘redox sensor’ (Sun et al. 1999) and may serve in this capacity to help modulate estrogen-responsive gene expression.

Because of their interdependent nature, it was somewhat surprising that Trx and TrxR would have different effects on cyclin G2, cyclin D1, and Bcl2 gene expression (Table 1). However, it is important to remember that estrogen-responsive genes are regulated not simply by ERα alone, but by a complex array of transcription factors and coregulatory proteins bound to multiple cis elements in extended gene regions, and that the association of a single transcription factor with a single gene region cannot necessarily be used to predict the transcriptional response.

### Biological roles of Trx and TrxR

Given the role of Trx and TrxR in influencing estrogen-responsive gene expression, it is not surprising that these two proteins would influence reproductive function. An earlier study suggested that Trx and TrxR were part of a uterine antioxidant system required for maintaining estrogen responsiveness of the uterus (Deroo et al. 2004). These and other studies have shown that E2 increases uterine expression of Trx and TrxR in rodents and humans (Maruyama et al. 1997, 1999, Osborne et al. 2001, Deroo et al. 2004). In addition to their roles in reproduction, Trx and TrxR have been implicated in cancer prevention (Urig & Becker 2006) and progression (Turunen et al. 2004, Biaglow & Miller 2005, Arner & Holmgren 2006, Fujino et al. 2006). The ability of Trx and TrxR to alter H2O2 levels (Fig. 6) could help to maintain gene expression by regulating the redox state of critical transcription factors. Furthermore, they could be essential in avoiding oxidative stress and limiting the damage to cellular macromolecules, which has been associated with aging and age-related disease.

### Table 1 Regulation of endogenous, estrogen-responsive genes in MCF-7 cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Trx</th>
<th>TrxR</th>
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<tbody>
<tr>
<td>pS2</td>
<td>↑</td>
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<tr>
<td>PR</td>
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<td>Cyclin G2</td>
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<td>Cyclin D1</td>
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<td>ERα</td>
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RNA interference experiments demonstrate the gene-specific effects of endogenously expressed Trx and TrxR on estrogen responsiveness.

### Figure 7 Oxidative stress response protein forms an interconnected network that alters ROS distribution and influences estrogen responsiveness.

Trx and TrxR reduce PDI (Lundstrom & Holmgren 1990); PDI prevents misfolding of many proteins including TrxR and SOD1 (Cheung et al. 1999, Atkin et al. 2006) and acts as a molecular chaperone for ERz (Schultz-Norton et al. 2006); and ERz associates with TrxR, PDI, SOD1, and Ape1 at endogenous estrogen-responsive genes (Fig. 3, Rao et al. 2008, Schultz-Norton et al. 2008, Curtis et al. 2009). Taken together, our studies suggest that ERz serves as a nucleating factor to recruit proteins involved in regulating oxidative stress to estrogen-responsive genes, and that oxidative stress proteins are, in turn, instrumental in altering estrogen-responsive gene expression and redox regulation.

**Declaration of interest**

None of the authors has a conflict of interest.

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