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The Exonuclease Activity of hPMC2 is Required for Transcriptional Regulation of The QR Gene and Repair of Estrogen-Induced Abasic Sites

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We have previously reported that the expression of antioxidative stress enzymes is upregulated by *trans*-hydroxytamoxifen (TOT) in breast epithelial cell lines providing protection against estrogen-induced DNA damage. This regulation involves Estrogen Receptor β (ERβ) recruitment to the Electrophile Response Element (EpRE) and a novel protein, human homolog of Xenopus gene which Prevents Mitotic Catastrophe (hPMC2). We have also demonstrated that ERβ and hPMC2 are required for TOT-dependent recruitment of poly (ADP-ribose) polymerase 1 (PARP-1) and Topoisomerase IIβ (Topo IIβ) to the EpRE. Sequence analysis reveals that the C-terminus of hPMC2 encodes a putative exonuclease domain. Using *in vitro* kinetic assays, we found that hPMC2 is a $3'$–$5'$ non-processive exonuclease that degrades both single-stranded and double-stranded substrates. Mutation of two conserved carboxylate residues drastically reduced the exonuclease activity of hPMC2, indicating the relative importance of the catalytic residues. Western blot analysis of breast cancer cell lines for Quinone Reductase (QR) levels revealed that the intrinsic exonuclease activity of hPMC2 was required for TOT-induced QR upregulation. Chromatin immunoprecipitation (ChIP) assays also indicated that hPMC2 was involved in the formation of strand breaks observed with TOT treatment and is specific for the EpRE-containing region of the QR gene. We also determined that the transcription factor NF-E2-related factor-2 (Nrf2) is involved in the specificity of hPMC2 for the EpRE. In addition, we determined that the catalytic activity of hPMC2 is required for repair of abasic sites that result from estrogen-induced DNA damage. Thus, our study provides a mechanistic basis for transcriptional regulation by hPMC2 and provides novel insights into its role in cancer prevention.

Introduction

Prolonged exposure to estrogen is strongly associated with an increased risk for developing breast cancer (Bolton and Thatcher, 2008). However, the precise role of estrogen in the initiation and progression of breast cancer has yet to be determined. Cytochrome P450 enzymes catalyze the oxidative metabolism of estrogens to hydroxy-catecholestrogens that is further oxidized to its semiquinone and quinone forms (Cavalieri *et al.*, 2006). The estrogen quinones can form unstable adducts with DNA, leading to depurination and mutations. Further, redox cycling between the quinone and semiquinone produces reactive oxygen species that accounts for oxidative damage to DNA and lipids associated with estrogen. The sustained oxidative stress and the mutagenic potential of these catechol estrogen quinones may contribute to the initiation while upregulation of mitogenic genes through the Estrogen Receptor (ER) may facilitate progression of breast cancer (Liehr, 2000; Yager and Davidson, 2006).

The Selective ER Modulator tamoxifen became the first drug to be approved for prophylactic use in the reduction of risk for breast cancer, showing a 50% reduction in both non-invasive and invasive breast cancer (Fisher *et al.*, 1998). Tamoxifen is believed to protect against breast cancer by blocking the ER-mediated transcription of mitogenic genes. However, we have previously determined that the antioxidative stress enzyme Quinone Reductase (QR) is upregulated by tamoxifen in breast epithelial cell lines (Montano and Katzenellenbogen, 1997; Montano *et al.*, 1998). QR is known to catalyze the reduction of estradiol-3,4-quinone preventing the generation of reactive oxygen species (Gaikwad *et al.*, 2007). QR expression is also increased in mammary glands of rats treated with tamoxifen and this correlates with a decrease in estrogen-induced DNA damage (Montano *et al.*, 2007).

Analysis of the QR transcriptional regulation indicates Estrogen Receptor β (ERβ) recruitment to the Electrophile Response Element (EpRE) and involves a novel protein, human homolog of Xenopus gene which Prevents Mitotic Catastrophe (hPMC2), also known as RNA exonuclease 4 homolog (REXO4) (Montano *et al.*, 1998, 2000). Our previous studies demonstrated a *trans*-hydroxytamoxifen (TOT)-dependent, localized co-recruitment of transcription factors ERβ, hPMC2, poly

(ADP-ribose) polymerase 1 (PARP-1) and Topoisomerase IIβ (Topo IIβ) to the EpRE of the QR gene (Sripathy et al., 2008). Lack of either ERβ or hPMC2 resulted in a complete loss of TOT-induced gene expression and loss of TOT-dependent decrease in levels of catechol estrogen quinones. The occupancy of NF-E2-related factor-2 (Nrf2) was not dependent on TOT; however, its recruitment was enhanced by TOT-liganded ERβ and hPMC2. Nrf2 is a transcription factor that is a regulator of antioxidative response while both PARP-1 and Topo IIβ are involved in DNA damage/repair and allow for transcriptional activation by relaxing chromatin (Nioi et al., 2003; Ju et al., 2006). Our previous results suggested that ERβ and hPMC2 are capable of assembling an activation complex at the EpRE in response to TOT. ERβ and hPMC2 also regulate expression and transcription of other antioxidative enzymes, such as Glutathione S-Transferase Pi (GSTpi) and gamma-Glutamylcysteine Synthetase Heavy Subunit (GCSh) that are dependent on the EpRE (Montano et al., 2004).

Our previous work had provided a mechanistic basis for the protective effects of TOT against estrogen-induced DNA damage and show that it is primarily mediated by ERβ and hPMC2 (Sripathy et al., 2008). ERβ has been suggested to have a protective role against breast cancer and its elevated levels of expression has been correlated with a positive response for tamoxifen treatment (Hopp et al., 2004; Nakopoulou et al., 2004; Saji et al., 2005). Additionally, our studies imply a crucial role for hPMC2 in mediating chemoprevention against breast cancer. However, not much is known about the biological role of this novel protein. Interestingly, the C-terminus of hPMC2 encodes a putative exonuclease domain and we speculated that this domain could have a role in transcriptional upregulation of QR.

Proteins that possess exonuclease domains are involved in a wide variety of cellular functions and have important roles in repair of DNA breaks, recruitment of DNA damage/repair factors as well as in transcriptional regulation (Moser et al., 1997). Work by Ju et al. (2006) has shown that the enzyme Topo IIβ associates itself with gene promoter regions and is required for site-specific double-strand break formation during receptor-mediated transcriptional activation. The double-strand break formation creates a signal resulting in activation

of PARP-1 and ultimately serves as a mechanism for initiation of gene transcription. This work thus mechanistically links the components of DNA damage and repair machinery to regulated gene transcription.

As a potential target for chemoprevention, it is important to understand the mechanism of action of hPMC2, in particular its exonuclease activity, subsequent to its recruitment at the EpRE. We provide supporting evidence that the exonuclease activity of hPMC2 is involved in transcriptional regulation of QR and in repair of estrogen-induced DNA damage.

Results

hPMC2 has a putative exonuclease domain

We have previously shown that hPMC2 is important for TOT-mediated protection against estrogen-induced DNA damage (Sripathy et al., 2008). However, not much is known about the biological role of this protein apart from its ability to increase transcription from EpRE-containing promoters of antioxidative stress enzymes in response to tamoxifen. As the C-terminus of hPMC2 encodes a putative exonuclease domain, the enzyme was placed in an exonuclease superfamily that includes RNases, the proofreading domains of the Pol I family of DNA polymerases and DNases such as Exo I (Moser et al., 1997). Homology within the superfamily is concentrated at three conserved motifs termed ExoI, ExoII and ExoIII. Nguyen et al. (2001) have characterized one of these exonucleases, Isg20 (interferon-stimulated gene product of 20 kDa), that cleaves both single-stranded RNA and DNA.

To compare Isg20 and hPMC2, we have performed an amino-acid alignment of the two proteins by COBALT (Constraint-based Multiple Alignment Tool) (Figure 1). The sequence alignment reveals that there is a significant homology between the two proteins. Work by Nguyen et al. (2001) has also shown that Isg20 is a 3′–5′ exonuclease, and site-specific mutations of D11 (in the ExoI motif), D94 (ExoII) and D154 (ExoIII) resulted in decreased exonuclease activities. Interestingly, these amino-acid residues are also conserved in hPMC2. To characterize the exonuclease activity of hPMC2 and to understand the role of the conserved amino-acid

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  1   --------------------------------------------------------------------------MAG    3
161   ERTNGDIVPERGDIEHKKRKAKEAAPAPPTEEDIWFDDVDPPDIEAAIGPEAAKIARKQLG-QSEGSVSLSLVKEQAFGG  239

  4   SREVVAIDCEMVGLGPH-RESGLARCSLVNVHGAVLYDKFIRPEGEITDYRTRVSGVTPQHMVGATPFAVARLEILQLLK   82
240   LTRALAIDCEMVGVGPKGEESMAARVSIVNQYGKCVYDKYVKPTEPVTDYRTAVSGIRPENLKQGEELEVVQKEVAEMLK  319

 83   GKLVVGHDLKHDFQALKEDMSGYTIYDTSTDRLLWREAKLDHCRRVSLRVLSERLLHKSIQNSLLGHSSVEDARATMELY  162
320   GRILVGHALHNDLKVLFLDHPKKKIRDTQKYKPF---KSQVKSGRPSLRLLSEKILGLQVQQA--EHCSIQDAQAAMRLY  394

163   RLHRKEFEQSIRTQNRKPEKTS--------  181
395   QI-----SQRIRARRGLPRLAVSD------    4
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Figure 1 Sequence alignment of Isg20 and hPMC2. The amino acid sequence of Isg20 was aligned with the C terminus of hPMC2. Numbers indicate the amino acid positions and gaps in the alignment are indicated by . The two sequences were aligned using COBALT from NCBI. The first line is the amino acid sequence of Isg20 while the next line indicates the sequence for hPMC2. Two conserved amino acids in hPMC2 (D247 and D386) enclosed by boxes are the residues that were mutated for the described studies.

residues in its enzymatic activity, we mutated two of these residues (D247G/D386G; Figure 1) and cloned it into a pET28 vector with an N-terminal His tag. The wild-type (WT) and the double mutant (DM) hPMC2 were purified as described in 'Supplementary methods' (Supplementary Figure 1).

Exonuclease activity of hPMC2

To determine the active site concentration of WT hPMC2, we performed multiple-turnover experiments, that is, several rounds of enzyme catalysis, in which the enzyme concentration is less than the substrate. Under these conditions, reaction of the single-stranded EpRE core with WT hPMC2 was characterized by an exponential burst of product formation, followed by a slower steady-state phase (Supplementary Figure 2). In such cases, the amplitude of the burst phase can be used to determine the amount of active enzyme. The kinetic experiments revealed that the purified WT hPMC2 was

either 50% active or existed as a dimer (Hsieh *et al.*, 1993).

To characterize the exonuclease activity of hPMC2, we performed single-turnover experiments, that is, one round of enzyme catalysis, in which enzyme concentration is in excess of the DNA substrate. These experiments were conducted with WT and DM hPMC2 and with either a single- or a double-stranded substrate containing the EpRE sequence.

With the single-stranded substrate, an increase in product formation was observed over the time course of the reaction (Figure 2a), indicating 3′–5′ exonuclease activity. The appearance of successively shorter 20-mers, 19-mers and so on is consistent with a distributive enzyme activity as opposed to a highly processive exonuclease (Skalski *et al.*, 2000). In addition, increasing the concentration of WT hPMC2 led to increased product formation along with an increase in the observed rate constants (Figure 2c; Table 1). With an increase from 100 to 500 nM WT hPMC2, the observed
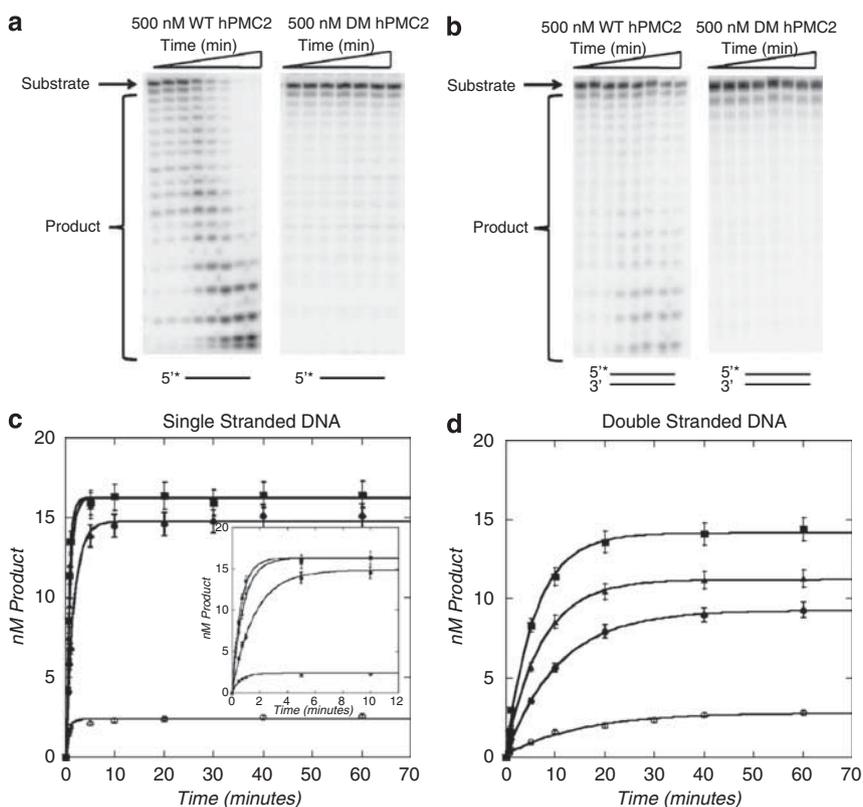

Figure 2 Representative autoradiograms and graphs of single and double stranded substrates with WT and DM hPMC2. Single turnover experiments, in which enzyme concentration is in excess of DNA, were performed with 20 nM single or double stranded EpRE substrate and increasing concentrations of either WT or DM hPMC2. The DNA was radioactively labeled on the 5′ end and increasing amounts of the enzyme was added and the reaction monitored for a period of 1 h. Gel images were obtained with a Molecular Dynamics Storm 840 phosphorimager system and data analysis was performed using ImageQuaNT software. The increase in product formation observed over the time course of the reaction was then plotted on KaleidaGraph. (**a**) Representative autoradiogram of three independent experiments of single stranded EpRE substrate with 500 nM WT or DM hPMC2. (**b**) Representative autoradiogram of three independent experiments of double stranded EpRE substrate with 500 nM WT or DM hPMC2. (**c**) KaleidaGraph of product formed over time with single stranded EpRE substrate at varying concentrations of WT or DM hPMC2: 100 nM (●), 300 nM (▲), 500 nM (■) WT hPMC2 and 500 nM (○) DM hPMC2. The inset shows the same plot over a shorter time period (12 min) to illustrate the difference in the rate constants at varying concentrations of the enzyme. (**d**) KaleidaGraph of product formed over time with double stranded EpRE substrate at varying concentrations of WT or DM hPMC2: 100 nM (●), 300 nM (▲), 500 nM (■) WT hPMC2 and 500 nM (○) DM hPMC2.

Table 1 The observed rate constants (k_{obs}) determined under single turnover conditions for single and double stranded EpRE substrates with increasing concentrations of WT hPMC2

WT hPMC2 concentration (nM)	k_{obs} (min^{-1})	
	Single stranded	Double stranded
100	0.62 ± 0.03	0.093 ± 0.004
300	1.25 ± 0.06	0.141 ± 0.008
500	1.62 ± 0.06	0.171 ± 0.010

Abbreviations: EpRE, Electrophile Response Element; WT hPMC2, wild type human homolog of Xenopus gene which Prevents Mitotic Catastrophe.

rate constant (k_{obs}) increased nearly 2.5-fold from 0.62 ± 0.03 to 1.62 ± 0.06 min^{-1}. With DM hPMC2, there was <10% product formation over a period of an hour and increasing concentrations of the enzyme did not lead to any increase in product formation (Figures 2a and c).

With the double-stranded EpRE substrate, the trend observed was similar to the single strand. The WT enzyme degraded the double-stranded substrate over the time course of the reaction, with increase in product formation observed with increased concentration of the enzyme (Figures 2b and d). With increase in WT hPMC2 from 100–500 nM, the observed rate constant (k_{obs}) increased nearly twofold from 0.093 ± 0.004 to 0.171 ± 0.010 min^{-1} (Table 1). As expected, there was insignificant product formation observed with DM hPMC2. However, with increase in concentration, hPMC2 degraded the single-stranded substrate more efficiently than the double-stranded EpRE substrate (Table 1). In fact, at 500 nM WT hPMC2 concentration, the enzyme degraded single-stranded DNA ~10-fold faster than the double-stranded DNA.

The catalytic activity of hPMC2 is required for TOT-induced QR expression

We have previously demonstrated a TOT-dependent recruitment of ERβ and hPMC2 to the EpRE, with subsequent recruitment of other factors to form a co-activator complex, resulting in transcriptional induction (Sripathy et al., 2008). This recruitment of a co-activator complex was observed in both the presence (MCF7 cell line) and absence of ERα (MCF10A-ERβ cell line, MCF10A cells stably transfected with ERβ). We also observed that recruitment of the complex to the QR promoter resulted in upregulation of QR expression (Sripathy et al., 2008). However, hPMC2 miRNA decreased expression of QR and other antioxidative stress enzymes, indicating that hPMC2 was crucial for the upregulation of antioxidative enzyme expression. From our kinetic studies reported above, we showed that the well-conserved exonuclease domain of hPMC2 is functional. As a result, we determined if the exonuclease activity had a role in TOT-induced QR expression.

We used both MCF7 as well as the MCF10A-ERβ cell lines to study TOT-induced QR expression. Both of these cell lines were transfected with either hPMC2 miRNA that targets hPMC2 3′-UTR or hPMC2 miRNA along with plasmid expressing WT or DM hPMC2. The cell lines were either untreated or treated with 10^{-6} M TOT for 3 h and analyzed via western blot for expression levels of QR and hPMC2. We confirmed that hPMC2 protein expression was downregulated in hPMC2 miRNA cells when compared with control MCF7 cells. In addition, we also confirmed that transfection of a plasmid expressing WT or DM hPMC2 in hPMC2 miRNA cells increased hPMC2 protein expression levels (Figure 3a). As previously reported, TOT treatment resulted in a significant increase in QR levels in control cells (Figure 3b). However, downregulation of hPMC2 using hPMC2 miRNA resulted in decreased basal QR expression and no induction of QR expression was evident after TOT treatment. The expression of WT hPMC2 was able to rescue TOT-induced QR expression to a significant level, while the exonuclease DM (D247G/D386G) was unable to rescue TOT-induced QR expression (Figure 3b).

In MCF10A-ERβ cells, the trend observed was similar to that observed in MCF7 cells. There is a significant increase in QR levels after treatment of control cells with TOT (Supplementary Figure 3). However, there is a decrease in QR levels after downregulation of hPMC2. In addition, the expression of WT hPMC2, but not DM hPMC2, was able to rescue TOT-induced QR expression to a significant level.

The exonuclease activity of hPMC2 is required for DNA strand breaks

Previous work by Ju et al. (2006) has shown that Topo II-mediated DNA strand break is required for regulated gene transcription. However, this was observed only on the ERE-containing region of the pS2 promoter in estrogen-treated MCF7 cells and not with TOT-treated cells. Previous work in our laboratory indicated a TOT-dependent recruitment of ERβ and hPMC2 to the EpRE, with subsequent recruitment of other factors including Topo IIβ to form a co-activator complex, resulting in transcriptional induction (Sripathy et al., 2008). Our experiments have proven that the exonuclease activity of hPMC2 is required for TOT-induced QR expression. As a result, we determined if hPMC2 had a role in the formation of DNA strand breaks during TOT treatment at the EpRE region of the QR gene.

DNA strand breaks were detected via 5-bromo-2′-deoxyuridine-5′-triphosphate (BrdUTP) labeling by terminal deoxynucleotidyl transferase (TdT). Chromatin immunoprecipitation (ChIP) assays were performed using anti-BrdU antibodies and PCR was performed to amplify the EpRE-containing region of the QR gene. We compared DNA strand break formation in MCF7 cells expressing hPMC2 miRNA, hPMC2 miRNA/WT hPMC2 and hPMC2 miRNA/DM hPMC2. Our results revealed that there was a threefold increase in DNA strand breaks after TOT treatment in control MCF7 cells (Figure 3c). However, when hPMC2 was down-
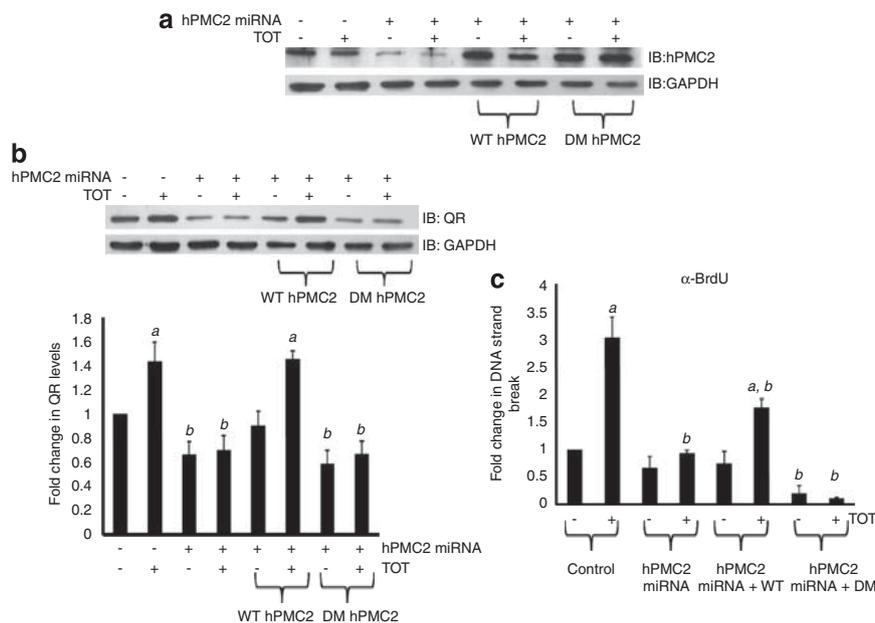
Figure 3 hPMC2 exonuclease activity is required for TOT induced QR expression and DNA strand breaks. MCF7 cells were transfected with either hPMC2 miRNA that targets hPMC2 3' UTR or hPMC2 miRNA along with plasmid expressing WT or the DM hPMC2. Cells were either untreated or treated with 10^{-6} M TOT for 3 h. (**a**) Proteins were extracted from cells and processed for western blot analyses of hPMC2 as described in 'Materials and methods'. Image shown is representative of three independent experiments. (**b**) Proteins were extracted from cells and processed for western blot analyses of QR as described in 'Materials and methods'. Levels of QR were quantitated and normalized to GAPDH. The gel image shown is representative of three independent experiments. Columns in the bar graph represent the fold change in QR levels in control or TOT treated samples. Error bars indicate s.e.m of three independent experiments. *a*, significance ($P < 0.05$) vs untreated cells; *b*, significance ($P < 0.05$) vs control transfected cells with the same treatment. (**c**) DNA strand breaks were detected by BrdUTP labeling by terminal deoxynucleotidyl transferase (TdT) and ChIP assays were performed using anti BrdU antibodies as described in 'Materials and methods'. The EpRE containing region of the QR gene was then amplified and the bars represent the fold change in DNA strand breaks at the EpRE in control or TOT treated samples. Error bars indicate s.e.m. of three independent experiments. *a*, significance ($P < 0.01$) vs untreated cells; *b*, significance ($P < 0.01$) vs control transfected cells with the same treatment.

regulated with miRNA, there was a decrease in DNA strand breaks to the basal level. Addition of a plasmid expressing WT hPMC2 partially compensated for this decrease while addition of the DM did not result in significant increase in strand breaks. This experiment indicates that the exonuclease activity of hPMC2 is involved in strand break formation observed with TOT treatment. In addition to the EpRE region of the QR gene, we also performed PCR amplification of the ERE-containing region of the ER target gene, pS2. The results indicated that TOT-induced strand breaks were specific for the EpRE region as strand breaks were not observed in the ERE region (Supplementary Figure 4).

As hPMC2 is involved in strand break formation, we determined if it has potential endonuclease activity. As a result, we performed endonuclease assays based on previous work by Ando *et al.* (2008). The assay was performed with either apurinic/apyrimidinic endonuclease I (APE1) or increasing concentrations of hPMC2 with supercoiled DNA containing the EpRE sequence as described in 'Supplementary methods'. Preliminary data indicates that while APE1 coverts the DNA into the nicked form, hPMC2 resolves it into nicked and linear forms suggesting endonuclease activity (Supplementary Figure 5).

Nrf2 is required for hPMC2 recruitment at the EpRE region of the QR gene

Transcription factor Nrf2 is essential for the antioxidant responsive element-mediated induction of phase II detoxifying and oxidative stress enzyme genes that respond to agents that cause oxidative stress (Nioi *et al.*, 2003). We have shown previously that while Nrf2 occupancy at the EpRE was not dependent on TOT, its recruitment was enhanced by TOT-liganded ERβ and hPMC2 (Sripathy *et al.*, 2008). In order to evaluate if the recruitment to the EpRE is specific to TOT, we performed ChIP assays with raloxifene. Raloxifene, like TOT, can induce QR and protect against oxidative damage (Bianco *et al.*, 2003). However, ChIP analysis in MCF7 cells reveals that raloxifene does not increase the occupancy of either hPMC2 or Nrf2 at the EpRE (Supplementary Figure 6A). It is possible that the structure of the ERβ-hPMC2 complex induced by the ligand is different due to difference in structures between TOT and raloxifene (Jordan, 1998; Osborne *et al.*, 2004).

Previous work has shown that downregulation of hPMC2 with miRNA in presence of TOT abrogated the enhanced recruitment of Nrf2 (Sripathy *et al.*, 2008). As this present work focuses on the role of the exonuclease activity of hPMC2, we were interested if it had a role in

Nrf2 recruitment. MCF7 cells were transfected with hPMC2 miRNA that targets hPMC2 3′-UTR along with plasmid expressing WT or DM hPMC2. The cells were either untreated or treated with 10⁻⁶ M TOT for 3 h and ChIP assays were performed for Nrf2 recruitment at the EpRE of the QR gene. Addition of WT hPMC2 increased Nrf2 recruitment, while the DM is incapable of inducing Nrf2 recruitment (Supplementary Figure 6B) at the EpRE. We have shown in this work that WT hPMC2 can cause DNA strand breaks at the EpRE while the DM is unable to do so (Figure 3c). It is possible to speculate that this inability to cause strand breaks by the DM prevents relaxation of chromatin thus preventing enhanced Nrf2 recruitment at the EpRE.

Another basis for TOT-induced recruitment of Nrf2 is upregulation of Nrf2 expression levels. Thus, a time course experiment was performed in MCF7 cells to determine the expression levels of Nrf2 in presence of TOT. Cells were either untreated or treated with 10⁻⁶ M TOT for 45 min, 90 min or 3 h, and Nrf2 levels were analyzed by western blots. This revealed a significant increase of Nrf2 levels at the 3 h time point (Supplementary Figure 7). We then downregulated Nrf2 with miRNA in MCF7 cells and the cells were either untreated or treated with 10⁻⁶ M TOT for 3 h and analyzed via western blot for expression levels of Nrf2. As expected, the Nrf2 levels increased with TOT treatment in control MCF7 cells (Figure 4a). When treated with miRNA, there was a significant drop in Nrf2 expression levels. To ensure the specificity of the miRNA against Nrf2, we checked the expression levels of transcription factors such as hPMC2, ERβ, PARP-1, Topo IIβ and RNA Pol II via western blot analysis. The results (Supplementary Figure 8) reveal that the levels

are unaffected, indicating the specificity of the miRNA against Nrf2.

Our ChIP assay results indicate that the TOT-induced DNA strand breaks were specific for the EpRE region of the QR gene. A possible factor influencing the recruitment of hPMC2 and thus DNA strand breaks in context of the EpRE but not the ERE is the presence of Nrf2 in the EpRE. Thus, we determined the effect of downregulation of Nrf2 on hPMC2 recruitment. The results revealed a significant increase in hPMC2 occupancy at the EpRE region on TOT treatment when compared with the control cells (Figure 4b). However, when Nrf2 levels were downregulated with miRNA, hPMC2 recruitment at the EpRE region was attenuated in both the presence and absence of TOT. This revealed that Nrf2 is required for hPMC2 recruitment at the EpRE region of the QR gene.

The catalytic activity of hPMC2 is required for removal of estrogen-induced abasic sites

An increase in the number of apurinic/apyrimidinic (AP) sites is a marker for oxidative DNA damage (Nakamura *et al.*, 2008). The AP sites resulting from oxidative damage to the deoxyribose moiety of DNA will lead to aldehydic forms of DNA lesions which, if not repaired, are promutagenic. Enzymes such as the human AP endonuclease (APE1) are known to catalyze the removal of abasic sites and avert potential mutagenic and cytotoxic effects of oxidative damage (Wilson *et al.*, 1995; Mol *et al.*, 2000).

Our previous work has shown that both ERβ and hPMC2 are required for TOT-mediated protection against oxidative DNA damage (Sripathy *et al.*, 2008).
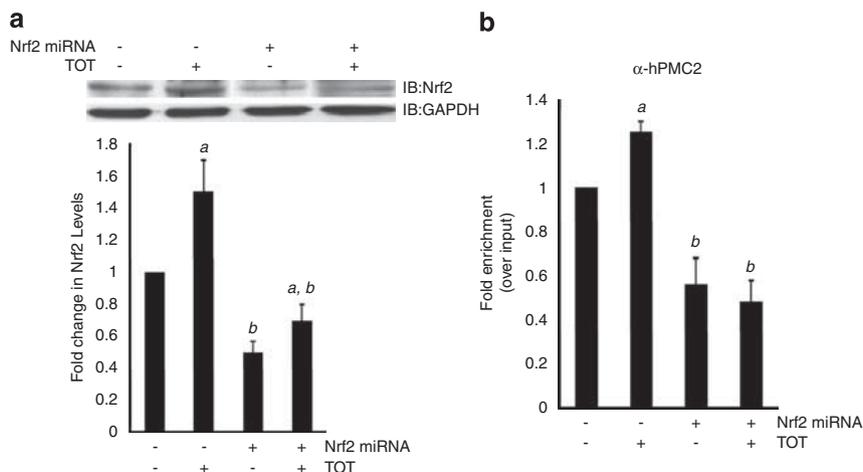

Figure 4 Nrf2 is required for hPMC2 recruitment at the EpRE region of the QR gene. (**a**) Nrf2 miRNA or control MCF7 cells were treated with 10⁻⁶ M TOT for 3 h. Proteins were extracted from cells and processed for western blot analyses of Nrf2 expression as described in 'Materials and methods'. Levels of Nrf2 were quantitated and normalized to GAPDH. The gel image shown is representative of three independent experiments. Columns in the bar graph represent the fold change in Nrf2 expression levels in control or TOT treated samples. Error bars indicate s.e.m. of three independent experiments. *a*, significance ($P < 0.05$) vs untreated cells; *b*, significance ($P < 0.01$) vs control transfected cells with the same treatment. (**b**) Nrf2 miRNA or control MCF7 cells were treated with 10⁻⁶ M TOT for 3 h and processed and analyzed using ChIP assays and hPMC2 antibody. The EpRE containing region of the QR gene was then amplified and the bars represent the mean of three replicate experiments for hPMC2 recruitment. Error bars indicate s.e.m. of three independent experiments. *a*, significance ($P < 0.01$) vs untreated cells; *b*, significance ($P < 0.01$) vs control transfected cells with the same treatment.

In addition to its exonuclease activity, we determined if hPMC2 attenuates estrogen-induced DNA damage by having a role in its repair. These experiments were carried out in ERα and ERβ negative-MCF10A cells thus allowing us to control for hPMC2-ERβ transcriptional regulation of antioxidative stress enzymes. Cells were transfected with either hPMC2 miRNA that targets hPMC2 3′-UTR or hPMC2 miRNA along with plasmid expressing WT or DM hPMC2. The cells were untreated or treated with E_2 (17-β estradiol) for 24 h and a biotinylated hydroxylamine aldehyde-reactive probe was used to detect the abasic sites as explained in 'Materials and methods'.

Our results revealed that in control MCF10A cells, treatment with E_2 resulted in a threefold increase in the number of AP sites, indicating that estrogen causes oxidative DNA damage (Figure 5). When hPMC2 was downregulated with miRNA, we observed an increase in number of AP sites not only in E_2-treated but also in untreated cells. To ensure that this was not due to hPMC2 inhibiting APE1, the major AP endonuclease involved in repair of abasic sites in the DNA (Wilson et al., 1995; Mol et al., 2000), we tested the level of expression of APE1. Western blot analysis reveals that the level of expression of APE1 is similar in both control and hPMC2 downregulated cells (Supplementary Figure 9). We also observed that addition of a plasmid expressing WT hPMC2 resulted in a decrease in number of AP sites to the basal level, while addition of the DM did not. This experiment thus indicates that the exonuclease activity of hPMC2 is required for repair of estrogen-induced abasic sites in DNA.
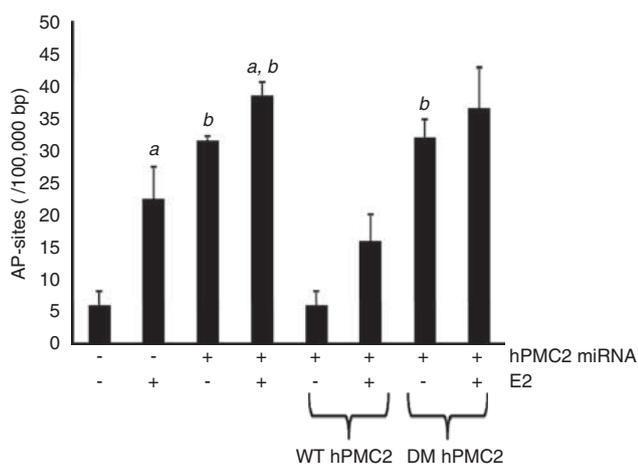

Figure 5 hPMC2 exonuclease activity is required for repair of estrogen induced abasic sites. MCF10A cells were transfected with either hPMC2 miRNA that targets hPMC2 3′ UTR or hPMC2 miRNA along with plasmid expressing WT or the DM hPMC2. Cells were either untreated or treated with 50 nM E_2 for 24 h. Genomic DNA was isolated from cells and processed for aldehyde reactive probe labeling as described in 'Materials and methods'. Columns represent the number of AP sites/10^5 bp in control or E_2 treated samples. Error bars indicate s.d. of two independent experiments. *a*, significance ($P<0.05$) vs untreated cells; *b*, significance ($P<0.05$) vs control transfected cells with the same treatment.

Discussion

Our studies provide evidence that hPMC2 has a 3′–5′ exonuclease activity and this activity is required for TOT-dependent QR upregulation and TOT-induced DNA strand breaks. These DNA strand breaks have been shown to trigger the recruitment of co-activator complexes to promoter regions and serve as a general mechanism for regulated initiation of gene transcription upon ligand stimulation (Ju et al., 2006). Our studies also indicate that Nrf2 is required for hPMC2 recruitment at the EpRE region of the QR gene. In addition, our present work reveals for the first time that the catalytic activity of hPMC2 is required for removal of estrogen-induced abasic sites in DNA.

Previous work has shown that hPMC2 is important for TOT-mediated protection against estrogen-induced DNA damage (Sripathy et al., 2008). Sequence analysis of hPMC2 indicates that there is a putative exonuclease domain at amino acids 233–406 that may provide clues regarding the cellular function of hPMC2 (Moser et al., 1997). Analysis of WT hPMC2 exonuclease activity with an EpRE-containing DNA substrate revealed that hPMC2 degrades DNA in a non-processive manner in the 3′–5′ direction. In addition, WT hPMC2 degrades single-stranded DNA nearly 10-fold faster than the double-stranded DNA. Interestingly, other exonucleases such as TREX1 (DNAse III) and p53 also exhibit non-processive excision in the 3′–5′ direction preferentially from single-stranded DNA (Huang, 1998; Hoss et al., 1999). These enzymes function as proofreading exonucleases for DNA polymerases in DNA repair processes (Skalski et al., 2000). It is thus possible to speculate that by virtue of its exonuclease activity, hPMC2 may also have a role in DNA repair.

To demonstrate the functional relevance of the exonuclease activity of hPMC2 in antioxidative gene expression, we downregulated hPMC2 protein expression and showed that decreased hPMC2 levels resulted in reduced QR levels. The expression of WT hPMC2 was able to rescue TOT-induced QR expression to a significant level unlike the DM (D247G/D386G). Therefore, our studies indicate a critical role for the exonuclease activity of hPMC2 in QR regulation.

Previous work by Ju et al. (2006) has shown that Topo II-mediated double-strand break is required for receptor-mediated gene transcription. We have already shown the TOT-dependent recruitment of ERβ and hPMC2 to the EpRE, with subsequent recruitment of other factors including Topo IIβ to form a co-activator complex, resulting in transcriptional induction (Sripathy et al., 2008). In addition, our ChIP analysis of BrdUTP-labeled cells indicates that hPMC2 exonuclease activity is required for DNA strand breaks resulting from TOT treatment.

While hPMC2 is recruited to the ERE after E_2 or TOT treatments; at the EpRE, we observe only TOT-dependent recruitment (Sripathy et al., 2008). One potential basis for the differential activation of EpRE vs ERE could be the presence of Nrf2 at the EpRE region. Our ChIP analysis in MCF7 cells with Nrf2 miRNA revealed that downregulation of Nrf2 results in

decreased recruitment of hPMC2 at the EpRE. This suggested that Nrf2 has a role in the recruitment of the ERβ–co-activator complex at the EpRE.

Proteins that possess exonuclease domains have important roles in the repair of DNA breaks, recruitment of DNA damage/repair factors as well as in transcriptional regulation (Moser *et al.*, 1997). The well-known APE1 possesses not only DNA repair activities of AP sites but also has a role in transcription by redox regulation of Jun binding activity (Xanthoudakis *et al.*, 1992). Similarly, p53 has sequence-specific DNA binding activity, which is required for transactivation functions and an exonuclease activity that may be involved in DNA repair (Marti and Fleck, 2004). However, the exonuclease activity of these proteins is separate from its ability to regulate transcription.

In this work, we have presented evidence that the exonuclease activity of hPMC2 is not only involved in transcriptional regulation but is also required for repair of estrogen-induced abasic sites in the DNA. While hPMC2-mediated regulation of QR and other antioxidative enzymes is crucial for TOT-ERβ protection, we have shown that hPMC2 also has a role in protection against DNA damage by reducing the number of AP sites. Our study thus provides evidence for a crucial role for hPMC2 in transcription as well as in DNA repair. In turn, this will provide important insights in the role of estrogen metabolites in breast tumor initiation and cancer prevention by hPMC2.

Materials and methods

Exonuclease assays

Single turnover experiments, where [Enzyme] > [DNA], were performed using either the single stranded or the double stranded EpRE substrate. In each case, the final DNA concentration was 20 nM with increasing concentrations of either WT or DM hPMC2. All kinetic reactions were performed at 37 °C, monitored for an hour and quenched at various time points with 100 mM EDTA. The aliquots were treated with 5 μl of formamide denaturing dye (90% formamide, 0.05% xylene cyanol and 0.05% bromophenol blue in TBE buffer) and were run on a 20% denaturing polyacrylamide gel in 1× TBE at 1600 V for 4 h. Gel images were obtained with a Molecular Dynamics Storm 840 phosphor imager system (Amersham Biosciences, Piscataway, NJ, USA) and data analysis was performed using ImageQuaNT software (version 4.2; Amersham Biosciences). The substrate and product bands on the gel were quantified and background in absence of enzyme was subtracted. The exonuclease products were plotted on KaleidaGraph version 3.0 (Synergy Software, Reading, PA, USA) and the observed rate constants were calculated (Berdis, 2001).

Active site concentration experiments were performed in the same manner as the single turnover assays using 200 nM single stranded EpRE DNA and 20 30 nM total protein. Appropriate fitting of the data using KaleidaGraph allowed for extraction of the active site concentration (Berdis, 2001).

Western blotting and quantitation

Whole cell lysates were processed and analyzed by western blotting as previously described (Sripathy *et al.*, 2008).

Chromatin immunoprecipitation

Cells were grown in 100 mm dishes and processed for ChIP analyses as previously described (Sripathy *et al.*, 2008) with minor modifications.

DNA strand break labeling and ChIP

Detection of DNA strand break formation was performed as previously described with slight modifications (Ju *et al.*, 2006) and ChIP was performed with an anti BrdU antibody (BD Biosciences, San Jose, CA, USA). The EpRE or the ERE containing regions of the QR or the pS2 gene, respectively, were amplified by PCR (Sripathy *et al.*, 2008).

Detection of AP sites

MCF10A cells were treated with either hPMC2 miRNA or hPMC2 miRNA along with a plasmid expressing WT or DM hPMC2. The cells were either untreated or treated with 50 nM E_2 for 24 h. DNA extraction was performed with a DNA isolation kit produced by Dojindo Molecular Technologies (Rockville, MD, USA). Aldehyde reactive probe labeling and quantification of abasic sites were performed with an AP sites assay kit (Dojindo Molecular Technologies) as previously described (Nakamura *et al.*, 2008).

Conflict of interest

The authors declare no conflict of interest.

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