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Original Contribution

Human OXR1 maintains mitochondrial DNA integrity and counteracts hydrogen peroxide-induced oxidative stress by regulating antioxidant pathways involving p21

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ABSTRACT

The oxidation resistance gene 1 (*OXR1*) prevents oxidative stress-induced cell death by an unknown pathway. Here, depletion of human OXR1 (hOXR1) sensitized several human cell lines to hydrogen peroxide-induced oxidative stress, reduced mtDNA integrity, and increased apoptosis. In contrast, depletion of hOXR1 in cells lacking mtDNA showed no significant change in ROS or viability, suggesting that OXR1 prevents intracellular hydrogen peroxide-induced increase in oxidative stress levels to avoid a vicious cycle of increased oxidative mtDNA damage and ROS formation. Furthermore, expression of p21 and the antioxidant genes *GPX2* and *HO-1* was reduced in hOXR1-depleted cells. In sum, these data reveal that human OXR1 upregulates the expression of antioxidant genes via the p21 signaling pathway to suppress hydrogen peroxide-induced oxidative stress and maintain mtDNA integrity.

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Introduction

The major sources of endogenous oxidative stress in the cell are reactive oxygen species (ROS), which are inevitably generated in mitochondria as by-products of aerobic metabolism.

ROS are also formed in other intracellular organelles, including cellular membranes, peroxisomes, and endoplasmic reticulum. For example, oxygen is reduced to superoxide by the NADPH oxidase complex in intracellular membranes such as granules in both inflammatory and noninflammatory cells [1]. Cells have evolved numerous mechanisms to counteract the toxic effects of ROS. For example, antioxidant enzymes such as catalase (CAT), superoxide dismutase, and glutathione peroxidase (GPX) detoxify ROS, whereas the base excision repair pathway removes oxidized bases from DNA in both mitochondria and nuclei [2]. Accumulation of DNA damage leading to mutations is implicated in diseases, including cancer and

neurodegeneration [3–5]. Mitochondrial dysfunction resulting from oxidative damage is thought to be the underlying cause of these pathologies, in which accumulation of oxidative mtDNA damage correlates with disease progression.

The eukaryotic oxidation resistance gene 1 (*OXR1*) is involved in protection against ROS and was identified by its ability to suppress mutagenesis in the DNA-repair-deficient *Escherichia coli* *nth mutH* mutant strain [6,7]. *OXR1* is a conserved gene family found in most eukaryotes, including worms, insects, and mammals, but is absent in prokaryotes. Mice lacking *Oxr1* display cerebellar neurodegeneration, suggesting a neuroprotective role for OXR1 that is especially important for postmitotic cells [8]. However, the mechanism underlying the prevention of oxidative damage is not clear. The cyclin-dependent kinase inhibitor 1A (p21) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) play important roles in controlling ROS levels by regulating antioxidant enzymes. Under basal conditions, the transcription factor Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), a substrate adaptor for the Cul3-dependent E3 ubiquitin ligase complex, which keeps Nrf2 levels low. p21 can stabilize and activate Nrf2 by competing with Keap1 for binding. Consequently,

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activated Nrf2 upregulates several genes, including the antioxidant enzymes GPX2, heme oxygenase-1 (HO-1), and NADH quinone oxidoreductase 1 (NQO1) [9].

In this paper we characterize the human (h) *OXR1* gene function by identifying isoforms and examining the maintenance of mitochondrial genome integrity and ROS balance. Cells depleted of hOXR1 showed reduced expression of *p21* and the antioxidant genes *HO-1* and *GPX2*, leading to increased levels of ROS, accumulation of mtDNA damage, and loss of mtDNA copy number during oxidative stress. Our data reveal that hOXR1 prevents hydrogen peroxide-induced oxidative stress to avoid a vicious cycle of increased mtDNA instability and increased ROS production.

Materials and methods

All methods and materials are described in detail in the supplementary information.

Small interfering RNA (siRNA) transfection

The siRNA oligos were transfected with Lipofectamine RNAi-MAX (Invitrogen).

MtDNA lesions measurement

Quantification of mtDNA lesions was monitored by the long-range PCR method and the TaqI-based qPCR method.

ROS measurement

General ROS were measured by flow cytometry of cells probed with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA).

Results

Identification of four major hOXR1 isoforms in human tissue

The human *OXR1* gene has been portrayed as a gene containing 16 exons in which the largest splicing form of 4465 bp encoded for a protein of 839 amino acids with a predicted molecular mass of 93.7 kDa (Accession No. NM_181354, identical to *hOXR1B* minus exon 14 in Fig. 1B) [10]. For clarity, in this report we have depicted *hOXR1* as a gene containing 19 exons (Fig. 1A). By searching the human expressed sequence tags (EST) database of the National Center for Biotechnology Information, we identified and sequenced a 4499-bp EST clone with an even longer putative open reading frame (873 amino acids) than *hOXR1B*, termed *hOXR1A* (Accession No. FN650108, Fig. 1B and C). Four major transcripts were detected by Northern blot in various human tissues (Fig. 1D). Isoforms A and C were expressed only in brain and testis, respectively, whereas the other two transcripts (isoforms B and D) were ubiquitously expressed (see Supplementary Fig. S1 and Supplementary Table S1 for more details).

RNA interference with hOXR1 sensitizes HeLa and 143B cells, but not 143B ρ 0 cells, to hydrogen peroxide

Elliott and Volkert [6] showed that the yeast *oxr1* mutant was sensitive to oxidative stress and that expression of hOXR1 in a yeast *oxr1* mutant reversed the sensitivity, suggesting an antioxidant stress function of hOXR1. To examine if hOXR1 protects human cells from oxidative stress, we used RNA interference to silence *hOXR1* in HeLa cells and subsequently exposed the cells to

hydrogen peroxide. Three different *hOXR1* siRNAs were tested in RNAi experiments (Fig. 2A). The *hOXR1_H6* and *_H8* siRNAs target exon 19, which is present in all isoforms, whereas *hOXR1_H10* siRNA targets exon 8, which is specific for the long isoforms *hOXR1A*, *B*, and *C* (Fig. 1B). First, the mRNA level of *hOXR1* was monitored by qPCR (with primers that detect expression of all isoforms) 2 days after siRNA transfection. The total transcription of *hOXR1* in knockdown cells was significantly reduced by all three *hOXR1* siRNAs, in which *hOXR1_H6* siRNA showed the strongest downregulation (85% reduction) compared to control cells (Fig. 2A). Furthermore, the protein level in hOXR1-depleted cells was examined by Western blot analysis using a commercial hOXR1 antibody raised against amino acid residues 175–225. The major hOXR1 protein band of about 100 kDa, corresponding to the long isoforms, was strongly reduced in HeLa cells after silencing with *hOXR1_H6* siRNA, confirming the efficiency of the RNA interference (Fig. 2B). Similarly, *hOXR1_H6* siRNA transfection also caused the reduction of hOXR1 protein at 100 kDa in HEK293, 143B, and 143B ρ 0 cells (Supplementary Fig. S2).

Next, we examined if *hOXR1* silencing sensitized HeLa cells to oxidative stress. Two days after transfection with *hOXR1* or control siRNA, cells were exposed to H₂O₂ for 1 h and allowed to recover for 2 days before cell viability was measured by the MTT assay. Cells depleted of hOXR1 from all three *hOXR1* siRNA transfections showed increased sensitivity to H₂O₂ (Fig. 2C), supporting a function of hOXR1 in counteracting oxidative stress in human cells. Further, hOXR1-depleted 143B and HEK293 cells also showed increased sensitivity to H₂O₂ (Fig. 2D). However, hOXR1-depleted 143B ρ 0 cells, which lack mitochondrial DNA, showed no increase in sensitivity to H₂O₂, suggesting that the function of hOXR1 in preventing oxidative stress depends on mtDNA in the cell.

Accumulation of mtDNA damage in hOXR1-depleted cells exposed to hydrogen peroxide

Previous reports suggest that hOXR1 localizes to the mitochondria. We tested the hypothesis whether hOXR1 may participate in repair of mtDNA. The integrity of mtDNA in cells exposed to H₂O₂ was examined by two different methods, either by assessing the ability to amplify a large template fragment by PCR (long-range PCR) or by analyzing the extent of restriction enzyme inhibition (TaqI-inhibition-based method). We showed that H₂O₂ readily damaged HeLa mtDNA in a concentration-dependent manner, confirming that cell death correlated with formation of mtDNA damage (Fig. 3A and C). It is evident from these experiments (Fig. 3A and C) that the TaqI inhibition-based method is most sensitive and precise for detection of lesions induced by sublethal doses of H₂O₂. Next, we monitored the level of mtDNA damage in control and hOXR1-depleted cells before and immediately after treatment and at 3 and 6 h of recovery. The untreated control and hOXR1-knockdown HeLa cells showed no significant difference in mtDNA integrity (Fig. 3B and D). In contrast, hOXR1-depleted cells accumulated significantly more mtDNA damage after exposure to H₂O₂ compared to control cells (Fig. 3B and D). After 3 and 6 h recovery, mtDNA integrity was the same as before treatment in hOXR1-depleted cells and control cells (Fig. 3B and D), suggesting that hOXR1 does not play a role in the repair of oxidative damage in mtDNA but renders mtDNA more susceptible to damage formed subsequent to oxidative stress.

Reduction of mtDNA copy number in hOXR1-depleted cells after hydrogen peroxide treatment

To investigate whether increased formation of mtDNA lesions in hOXR1-knockdown cells affects the stability of mtDNA, we measured the copy number of mtDNA in hOXR1-depleted cells and

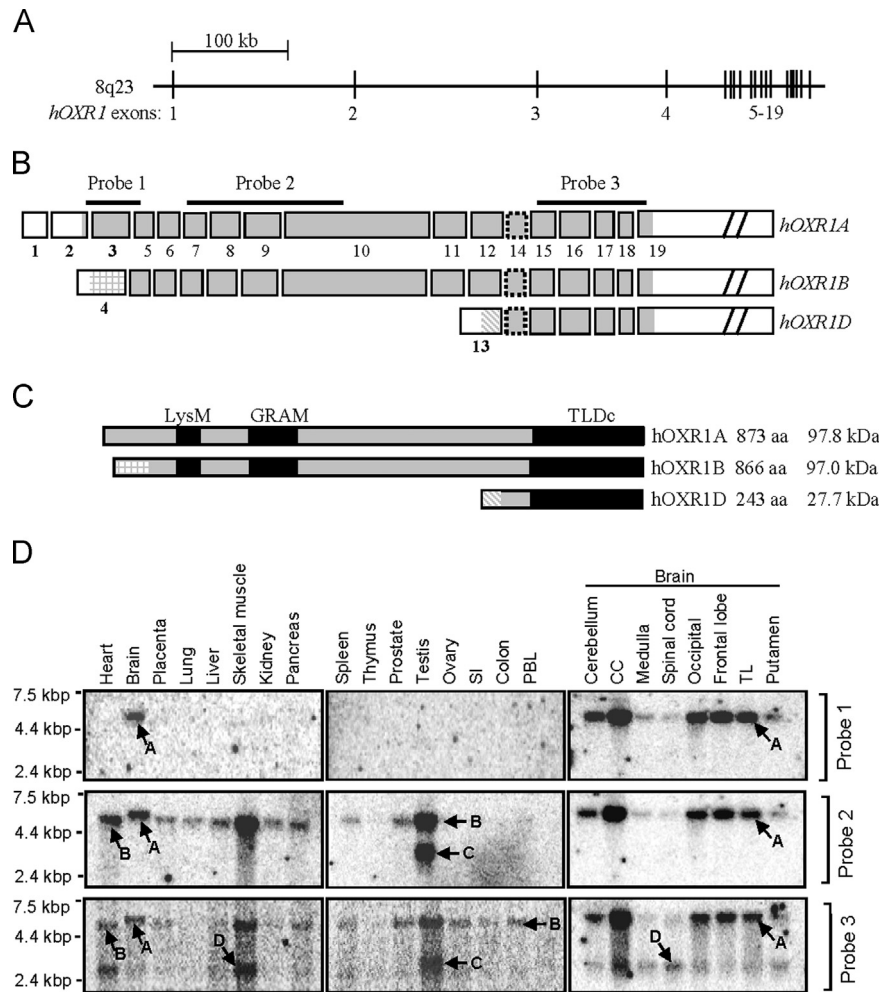


Fig. 1. Genomic structure and isoforms of human OXR1. (A) Genomic structure of *hOXR1*. The *hOXR1* gene is located on chromosome 8q23, contains 19 exons, and spans 547,883 bp. The intron length between exons is shown to scale. (B) Exon organization of *hOXR1* isoforms. The exon lengths are shown to scale except the untranslated part of exon 19, which is indicated by a double slash. White boxes indicate untranslated regions; gray boxes indicate open reading frames; dashed lines indicate exon 14, which is an alternative exon in isoforms *hOXR1A*, *B*, *C*, and *D*. The boxes marked with lattice and stripes indicate the unique sequence organization in isoforms *hOXR1B* and *D*, respectively. The positions of probes used for Northern analysis are indicated. (C) Predicted peptides and conserved domains of *hOXR1* isoforms. All isoforms contain the TLDc (IPR006571) domain, whereas isoforms *A* and *B* contain the additional LysM (IPR002482) and GRAM (IPR004182) domains. The unique sequences of *hOXR1B* and *hOXR1D* are marked in lattice and stripes, respectively. The predicted numbers of amino acids (aa) of the isoforms containing exon 14 and their molecular masses (kDa) are indicated. (D) Identification of *hOXR1* isoforms by Northern blot analysis. Three different human multiple-tissue poly(A)⁺ RNA blots were hybridized with ³²P-labeled *hOXR1* probes 1–3. The membranes were stripped after each cycle of hybridization. Arrows A–D indicate *hOXR1* transcripts A–D, respectively. SI, small intestine; PBL, peripheral blood leukocytes; CC, cerebral cortex; TL, temporal lobe.

control cells exposed to H₂O₂ (Fig. 3E). In untreated cells, the mtDNA copy number of *hOXR1*-knockdown cells was slightly, but significantly, reduced compared to the control cells. Notably, the copy number in control cells was restored completely after 6 h recovery, whereas the copy number in *hOXR1*-depleted cells was still not restored after 24 h recovery. This indicates that replication of mtDNA is delayed in the *hOXR1*-depleted cells.

Increased oxidative stress in the absence of *hOXR1*

To examine the role of *hOXR1* in regulating the intracellular ROS level, we used dichlorodihydrofluorescein diacetate, which is oxidized by several one-electron-oxidizing species, such as hydroxyl radicals ([•]OH), hydrochlorous acid (HClO), and reactive species formed from the decomposition of peroxynitrite (ONOO⁻/ONOOH) [11]. First, we measured ROS levels in untreated HeLa cells or immediately after exposure to H₂O₂ at two different concentrations. As shown in Fig. 4A, the ROS level increased in a dose-dependent manner in both control and *hOXR1*-depleted HeLa cells. Before exposure to H₂O₂, ROS levels, measured as the

mean of DCF fluorescence, were about 2.5-fold higher in *hOXR1*-depleted HeLa cells compared to control cells (Fig. 4A). After exposure to 0.25 and 0.5 mM H₂O₂ the difference between *hOXR1*-depleted HeLa and control cells was even higher (Fig. 4A). In the next set of experiments we examined the significance of *hOXR1* for ROS production in cells lacking mtDNA. We compared ROS levels in the isogenic cell lines 143B and 143B ρ 0, of which 143B ρ 0 cells lack mtDNA. In line with the results in HeLa, we find that ROS levels increased significantly in *hOXR1*-depleted 143B cells compared to control cells (Fig. 4B). In contrast, we observed no significant difference in ROS level between *hOXR1*-depleted and control 143B ρ 0 cells (Fig. 4C), suggesting that *hOXR1* is important for suppressing hydrogen peroxide-induced oxidative stress generated as a consequence of mtDNA damage.

Intracellular superoxide is independent of *hOXR1*

Superoxide anion is mainly generated by mitochondrial complexes I, II, and III as electrons bypass the normal electron transport chain and react directly with molecular oxygen [12]. To determine whether the mtDNA-dependent ROS generation in *hOXR1*-depleted cells is due to

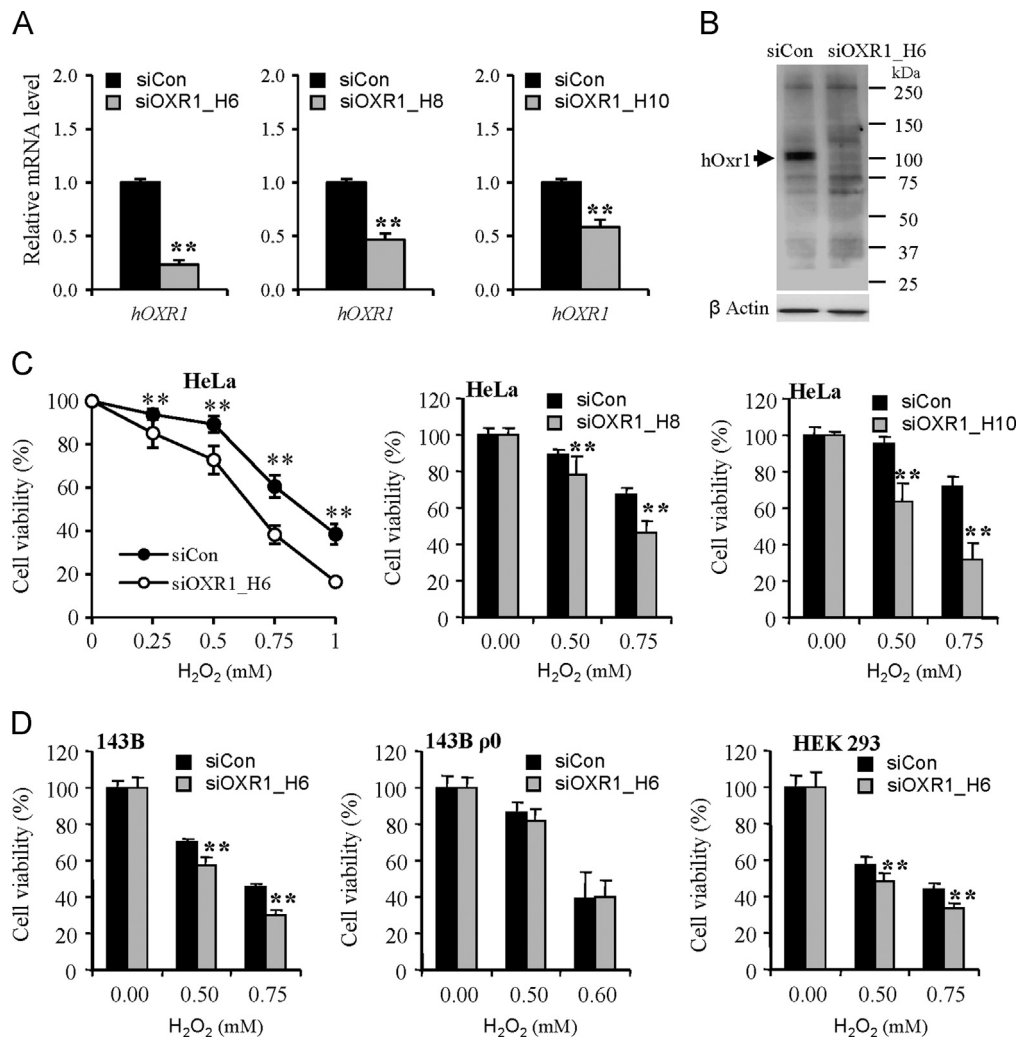


Fig. 2. Increased sensitivity to hydrogen peroxide in hOXR1-depleted HeLa, HEK293, and 143B, but not 143B ρ 0 cells. (A) Relative *hOXR1* mRNA 2 days after transfection with H6, H8, and H10 siRNA in HeLa cells. The standard deviations were calculated from two experiments, each with duplicate samples. (B) Western blot analysis of HeLa cell extract ($\sim 30 \mu\text{g}$) with anti-hOXR1 and β -actin antibody. (C and D) Cells were transfected with siRNA and, after 2 days, treated with increasing concentrations of hydrogen peroxide for 1 h and recovered in standard medium for 48 h. Cell viability was measured by the MTT assay. The standard deviation was calculated from three independent experiments, each with six samples. siCon, control siRNA; siOXR1_H6, H8, H10, hOXR1_H6, H8, H10 siRNA; ** $P < 0.01$.

superoxide anion, we measured the level of cellular superoxide level using the MitoSOX probe in HeLa, 143B, and 143B ρ 0. There were no significant differences in the basic levels of superoxide between hOXR1-depleted 143B and 143B ρ 0 cells compared to control cells (Supplementary Fig. S3). Menadione is known to enhance the superoxide production by redox cycling within mitochondria [13]. As expected, we measured increased concentrations of superoxide in HeLa and 143B cells after menadione treatment, but not after treatment with H₂O₂ (Fig. 4E and F). Menadione did not induce superoxide in 143B ρ 0 cells (Fig. 4G). Notably, we observed no significant differences in the levels of menadione-induced superoxide between hOXR1-depleted and control cells (HeLa and 143B cells; Fig. 4E and F), suggesting that mitochondrial superoxide production does not contribute to increased ROS in the absence of hOXR1. In agreement with these data, depletion of hOXR1 did not affect the cell viability after menadione treatment (Fig. 4D). Thus, it appears that hOXR1 regulates a subset of intracellular ROS, which does not include superoxide.

Depletion of hOXR1 induces apoptosis

To further investigate the protective role of hOXR1 in oxidative stress, we quantified the ratio of apoptotic cells by flow cytometry

after staining cells with propidium iodide and Fluor 488-conjugated annexin V. hOXR1-depleted HeLa cells exposed to 0.5 mM H₂O₂ showed an about two-fold increase in apoptosis compared to control cells (Fig. 4H). Taken together, our data indicate that depletion of hOXR1 increases the hydrogen peroxide-induced oxidative stress, reduces mtDNA integrity, and consequently triggers cell death via apoptosis.

Depletion of hOXR1 downregulates expression of p21 and the antioxidants GPX2 and HO-1

p21 is a well-characterized tumor suppressor involved in numerous processes including cell oxidative stress defense and apoptosis, which are affected in hOXR1-depleted cells. To investigate a possible link between p21 and hOXR1, we examined the expression level of p21 by qPCR in four different cell types (HeLa, HEK293, 143B, and 143B ρ 0). The results showed that p21 was strongly reduced in HeLa cells after hOXR1 depletion by each of three different *hOXR1* siRNAs (Fig. 5A). Similar downregulation of p21 was shown by *hOXR1* silencing in three other cell types, HEK293, 143B, and 143B ρ 0 (Supplementary Fig. S4), suggesting that basal expression of p21 depends on hOXR1. Next, we examined whether hOXR1 regulates p21 during exposure to hydrogen peroxide. We found that the

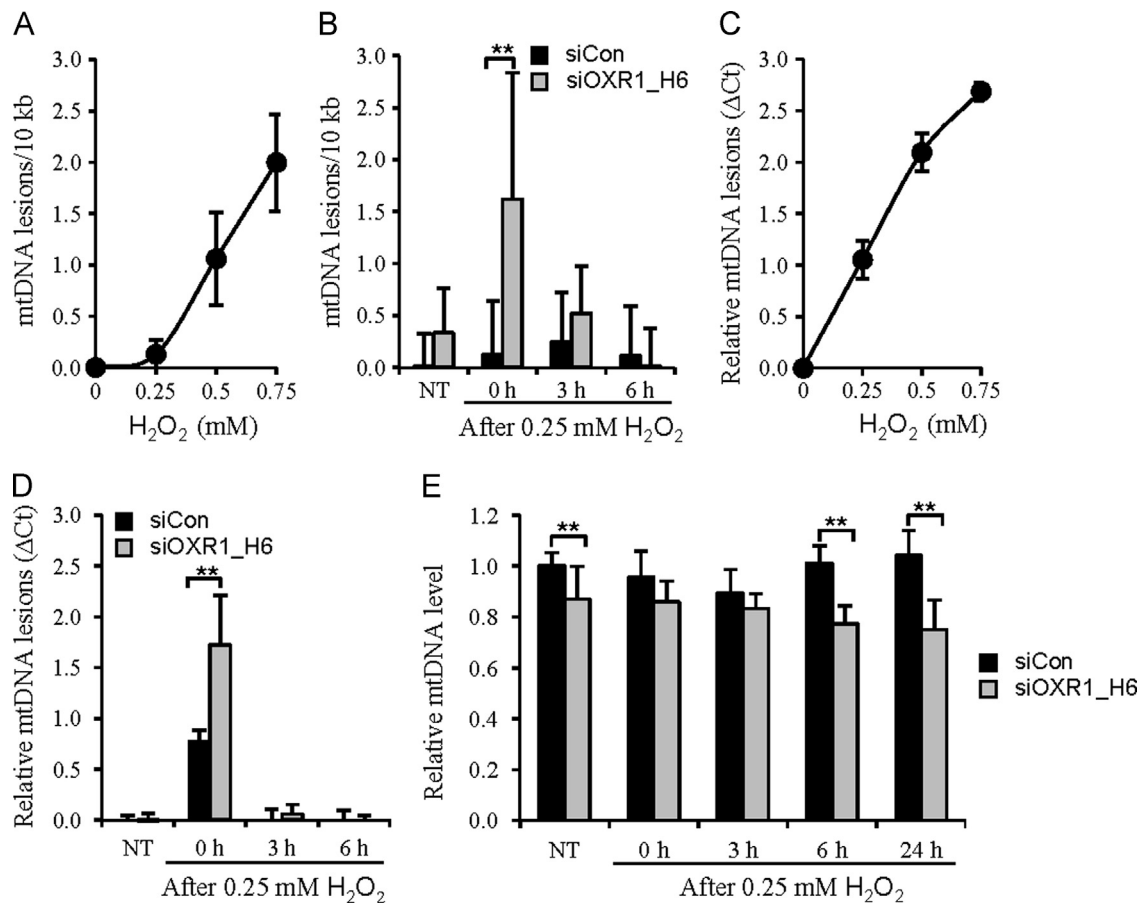


Fig. 3. Increased formation of mtDNA damage and reduced mtDNA copy number in hOXR1-depleted HeLa cells. (A) Dose-dependent accumulation of H₂O₂ induced mtDNA lesions. HeLa cells were treated with the indicated doses of H₂O₂ for 1 h and harvested immediately. Total DNA was isolated and the frequency of mtDNA lesions was determined by the ability to amplify a large PCR product. (B) H₂O₂-induced mtDNA lesions. The cells were treated with 0.25 mM H₂O₂ for 1 h after recovery in standard medium at the indicated time points. Total DNA was isolated and the frequency of mtDNA lesions was determined by long-range PCR. (C) Dose-dependent accumulation of H₂O₂-induced mtDNA lesions was measured using the TaqI inhibition-based method. The same DNA samples as used in (A) were analyzed. (D) H₂O₂-induced mtDNA lesions were measured by the TaqI inhibition-based method. The same DNA samples as used in (B) were analyzed. (E) Effect of H₂O₂ exposure on mtDNA copy number. siRNA-transfected HeLa cells were exposed to H₂O₂ for 1 h followed by recovery in standard medium for the indicated time points. Total DNA was isolated and mtDNA copy numbers were determined by qPCR. The standard deviation (in A–E) was calculated from two independent experiments, each with four samples. NT, nontreated cells; siCon, control siRNA; siOXR1_H6, hOXR1_H6 siRNA; ***P* < 0.01 compared with its control.

relative induction of *p21* during oxidative stress was independent of hOXR1 (Supplementary Fig. S5). Western analysis of extracts from hOXR1-depleted cells showed that the *p21* protein level was strongly reduced in untreated cells, and importantly, the increased gene expression upon oxidative stress did not result in increased *p21* protein levels in hOXR1-depleted cells (Fig. 5B and Supplementary Fig. S6).

Increased hydrogen peroxide induced-oxidative stress in hOXR1-depleted cells is indicative of a role in ROS detoxification. When gene expression of several antioxidant genes was analyzed in hOXR1-depleted cells, we found that *HO-1* and *GPX2* mRNA levels were downregulated under both basic level and oxidative stress conditions compared to control cells (Fig. 5C and Supplementary Fig. S5), whereas the genes *CAT*, *NQO1*, and *GPX1* were unaffected (Supplementary Fig. S5). Chen and colleagues showed that *p21* positively regulates Nrf2 by interfering with Keap1-mediated ubiquitination to avoid protein degradation of Nrf2 [9]. Nrf2 upregulates expression of antioxidant genes such as *GPX2* and *HO-1* in HCT116 cells and murine embryonic fibroblasts. We confirmed that *HO-1* expression was significantly downregulated in *p21*- and Nrf2-depleted HeLa cells (Fig. 5D and E), supporting the finding by Chen et al. [9]. However, *HO-1* expression was similar in *p21*-depleted cells and control cells upon H₂O₂ treatment (Fig. 5F). Furthermore, the basal level of *GPX2*

expression was upregulated in *p21*- and Nrf2-depleted cells (Fig. 5D and E) but downregulated after H₂O₂ treatment in *p21*-depleted cells (Fig. 5F). It thus appears that regulation of *HO-1* and *GPX2* by the *p21*-Nrf2 signaling pathway depends on the stress condition. Finally, depletion of either *HO-1* or *GPX2* increased ROS in HeLa cells (Supplementary Fig. S7), supporting an important function of *GPX2* and *HO-1* in HeLa cells to protect against ROS. In conclusion, these data suggest that hOXR1 prevents hydrogen peroxide-induced oxidative stress by positively regulating *GPX2* and *HO-1* expression, partly via the *p21*-Nrf2 signaling pathway.

Discussion

In this report, we have identified four major isoforms of hOXR1 (A–D). RNAi-mediated silencing of *hOXR1* increased sensitivity to hydrogen peroxide and showed that hOXR1 prevents formation of mtDNA damage and loss of mtDNA copy number induced by oxidative stress. Further, the results demonstrated that hOXR1 plays a role in detoxifying cellular ROS by upregulating gene expression of the antioxidants *GPX2* and *HO-1*, partly through the *p21*-Nrf2 pathway (summarized in Fig. 6). Thus it appears that hOXR1 is a key regulator in preventing hydrogen peroxide-induced oxidative stress and maintaining mtDNA integrity in the cell.

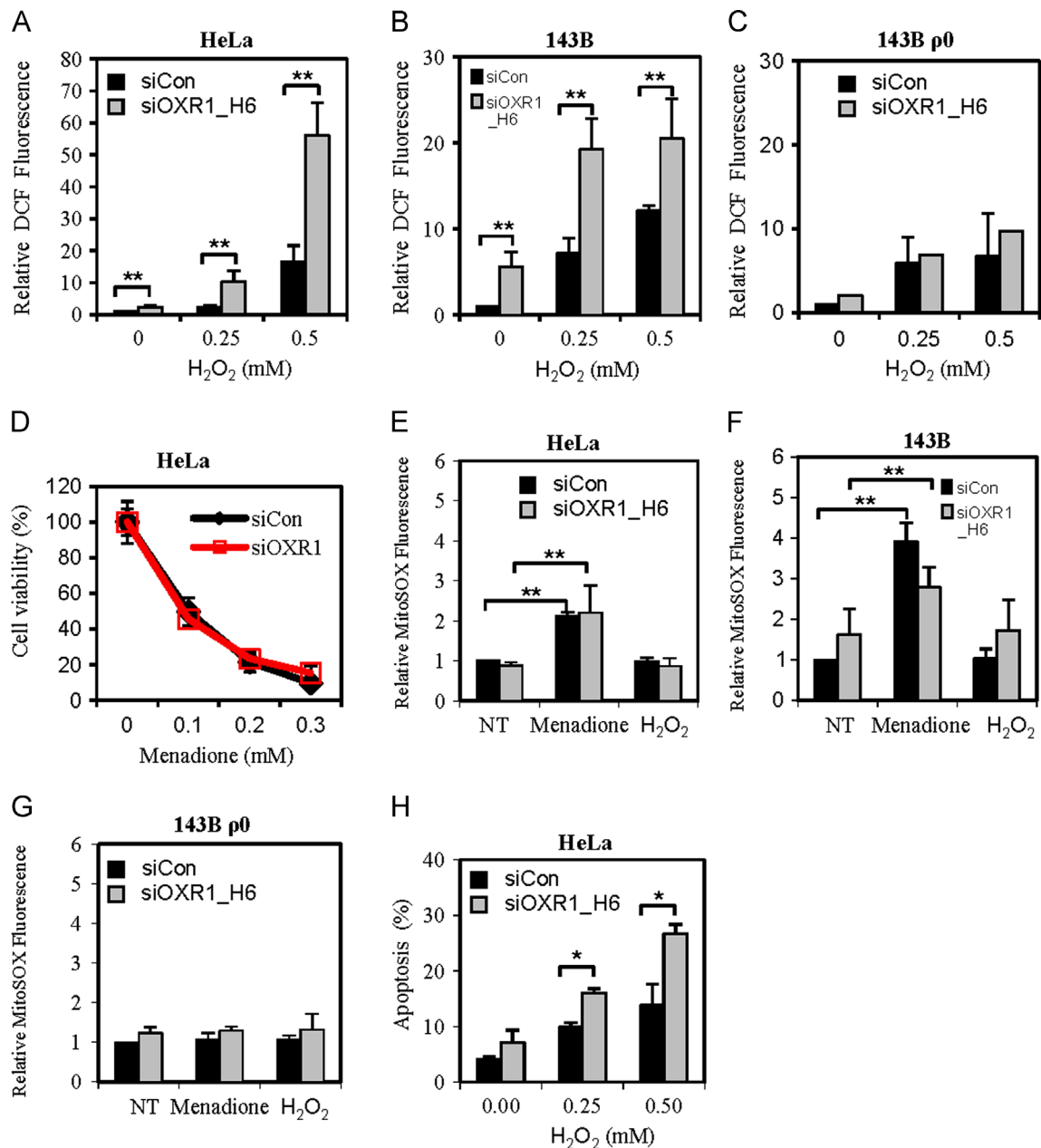


Fig. 4. Human OXR1 suppresses H₂O₂-induced oxidative stress in an mtDNA-dependent manner. (A) HeLa, (B) 143B, or (C) 143B ρ0 cells were preincubated with 5 μM CM-H₂DCFDA probe for 30 min, exposed to H₂O₂ for 1 h, and collected for flow cytometry. The average values of three independent experiments, each with duplicate samples, are shown. (D) hOXR1 depletion did not affect cell viability after menadione treatment. The HeLa cells were transfected with control siRNA or hOXR1_H6 siRNA and, after 2 days, treated with menadione for 1 h and recovered in normal Dulbecco's modified Eagle's medium for 48 h. The cell viability was measured by MTT assay. (E–G) The cells were exposed to 0.05 mM (HeLa) or 0.1 mM (143B and 143B ρ0) menadione for 1 h, or exposed to 0.25 mM H₂O₂ for 1 h, and stained with 3 μM MitoSOX (probe for superoxide) for 10 min and analyzed by flow cytometry. (H) H₂O₂-induced apoptosis increased in hOXR1-depleted HeLa cells. The cells were treated with or without H₂O₂ for 1 h and allowed to recover in normal medium for 24 h. The cells were stained with propidium iodide and Fluor 488-conjugated annexin V for 15 min and analyzed by flow cytometry. The standard deviation was calculated from three independent experiments, each with duplicate samples. siCon, control siRNA; siOXR1_H6, hOXR1_H6 siRNA; **P* < 0.05, ***P* < 0.01.

The *OXR1* gene was identified based on its antimutator activity in an *E. coli* (*mutH nth*) mutant deficient in oxidative DNA damage repair [7]. The role of OXR1 in preventing oxidative stress-induced cell death has been previously addressed in yeast, mosquitoes, and mice [7,8,14,15]. Expression of truncated human *OXR1* in yeast reversed H₂O₂ sensitivity in yeast *oxr1* mutants, suggesting a similar function in humans [6]. In this paper, based on the *hOXR1*-knock-down experiments in several human cell lines, we demonstrated that *OXR1* has the same antioxidation function in human cells as observed in model organisms. In addition, overexpression of green fluorescent protein-targeted hOXR1A increased the resistance to

hydrogen peroxide and induced *p21* mRNA expression in HeLa cells (Supplementary Fig. S8), supporting the notion that OXR1 protect against oxidative stress in a p21-dependent manner. Moreover, we showed that depletion of hOXR1 causes an increase in mitochondrial DNA instability and apoptosis, consistent with the finding by Oliver and co-workers [8] that mice lacking *Oxr1* displayed enhanced apoptosis in the cerebellar granule cell layer. In HeLa cells exposed to a sublethal dose of hydrogen peroxide, mtDNA was repaired within 3 h in both normal and hOXR1-depleted cells, suggesting that DNA repair capacity is not affected. However, the mtDNA copy number was not restored within 24 h

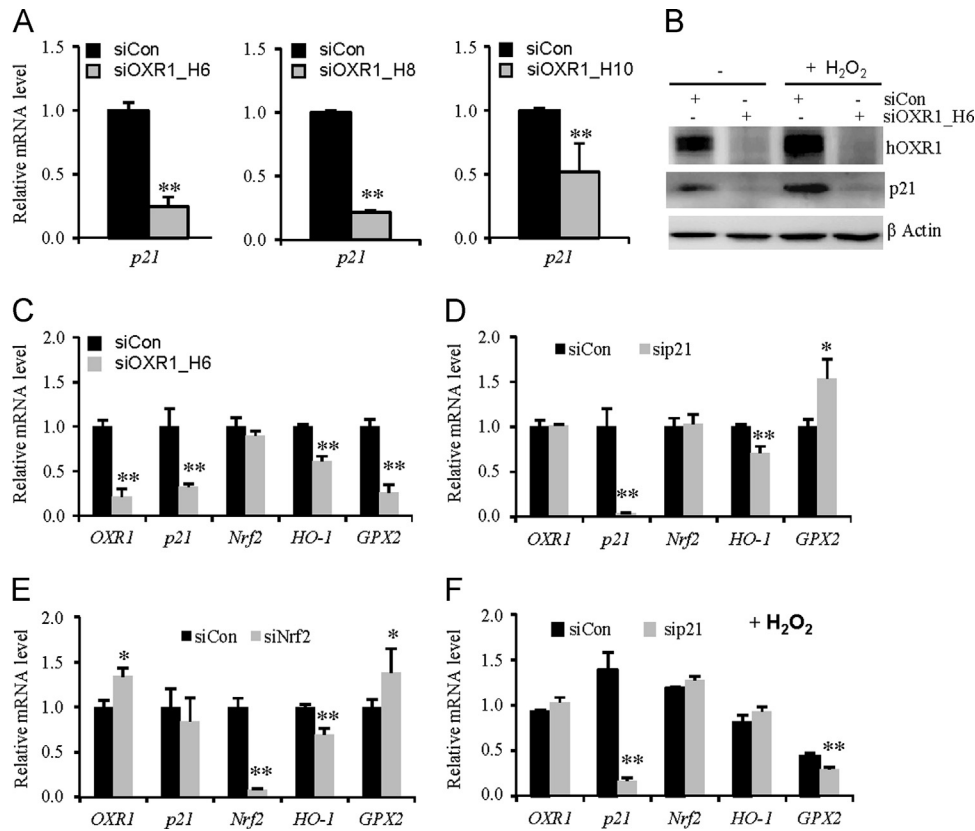


Fig. 5. hOXR1 depletion reduced expression of *p21*, *HO-1*, and *GPX2*. (A) *p21* mRNA was measured by qPCR 2 days after transfection of HeLa cells with control siRNA or hOXR1_H6, H8, and H10. *GAPDH* was used as internal control. The standard deviation was calculated from three independent experiments, each with duplicate samples. (B) Western blot analysis of hOXR1 and *p21*. β -Actin was used as loading control. HeLa cells were treated with or without 0.25 mM H_2O_2 for 1 h and allowed to recover in standard medium for 24 h. (C–E) Two days after HeLa cells were transfected with the indicated siRNA, total RNA was isolated and the levels of the indicated genes were measured by qPCR. The standard deviation was calculated from three independent experiments, each with duplicate samples. siCon, control siRNA; siOXR1_H6, hOXR1_H6 siRNA; sip21, *p21* siRNA; siNrf2, *Nrf2* siRNA. * $P < 0.05$, ** $P < 0.01$. (F) Two days after HeLa cells were transfected with *p21* siRNA, the cells were exposed to 0.5 mM hydrogen peroxide for 1 h and allowed to recover for 20 h. Total RNAs were isolated and the cDNA of the indicated genes was measured by qPCR. The standard deviation was calculated from three independent experiments, each with duplicate samples. siCon, control siRNA; sip21, *p21* siRNA. ** $P < 0.01$.

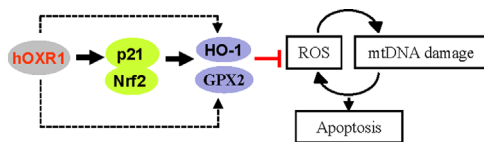


Fig. 6. Model of hOXR1-mediated antioxidant pathways. In this model hOXR1 inhibits oxidative mtDNA damage-associated ROS by positively regulating antioxidant *GPX2* and *HO-1* expression via *p21*-*Nrf2*-dependent (in solid lines) and independent pathways (in dotted lines). Thus, hOXR1 controls ROS levels to maintain mitochondrial genome stability and prevents apoptosis.

in hOXR1-depleted cells, indicating increased degradation of mtDNA and/or delayed replication.

The biochemical function of hOXR1 is still largely unknown. Depletion of hOXR1 did not affect mtDNA repair capacity, ruling out a role in mtDNA repair. Recently, Oliver and co-workers showed that mouse *Oxr1* was oxidized by H_2O_2 on the same order of magnitude as bovine serum albumin, arguing against a role in direct scavenging of ROS [8]. In the mosquito *Anopheles gambiae*, a role for OXR1 in transcriptional regulation of *CAT* and *GPX1* has been proposed [14]. The cerebellum of mice lacking *Oxr1* also displayed a significant reduction in *Gpx1* expression [8]. In our studies, depletion of hOXR1 in HeLa cells had no effect on the regulation of *CAT* or *GPX1*, but the mRNA level of the antioxidants *GPX2* and *HO-1* was significantly reduced in hOXR1-depleted cells. We also performed Western blot for *HO-1* and *GPX2* in HeLa and 143B cells. However, we failed to detect these two proteins in either normal or siRNA-transfected cells, suggesting that the expression level is too

low to be detected. We showed that hOXR1 partly regulates *HO-1* and *GPX2* via the *p21*-*Nrf2* pathway. In contrast, depletion of hOXR1 in HCT116 cells did not change the transcription level of hOXR1 in HCT116 cells did not change the transcription level of *p21* or *GPX2*. HCT116 cells were not sensitized by hOXR1 depletion, either (Supplementary Fig. S9), thereby supporting the idea that upregulation of *p21* and *GPX2* via hOXR1 is important for cell survival during oxidative stress. Thus, it seems that the OXR1 function has evolved a role in regulating antioxidant pathways, which is species- and cell-dependent.

Our data reveal that the expression of *p21* is regulated in a hOXR1-dependent manner. However, the differences we find in transcript levels of *HO-1* and *GPX2* by depleting hOXR1 and *p21* indicate that hOXR1 also regulates gene expression via a *p21*-independent pathway: Depletion of hOXR1 caused a reduction in transcription of both *HO-1* and *GPX2* under both normal conditions (Fig. 5C) and stress (Supplementary Fig. S5). In contrast, depletion of *p21* under normal conditions downregulated only *HO-1* and not *GPX2* (Fig. 5D). Under stress *GPX2* was downregulated in *p21*-depleted cells, but not *HO-1* (Fig. 5F).

Depletion of hOXR1 results in a large increase in ROS (Fig. 4A and B), suggesting hOXR1 plays a crucial role in preventing hydrogen peroxide-induced oxidative stress. To further test the hypothesis, we also co-incubated cells with the antioxidant NAC (*N*-acetylcysteine; 1 mM) for 1 h during exposure to 0.75 mM hydrogen peroxide. The result showed that co-incubation with NAC rescued the cell viability up to the same level as in the control cells in hOXR1-depleted HeLa cells (Supplementary Fig. S10). Thus, hOXR1 prevents oxidative stress-induced cell death by reducing

cellular ROS. Regarding the functional domains of hOXR1, Murphy and Volkert [16] suggest that “exon 8” (corresponding to exon 11 of hOXR1A in Fig. 1B) is important for the antioxidation function. In contrast, another study showed that the conserved TLDC domain in the C-terminal of mouse Oxr1 (similar to hOXR1D) is sufficient to functionally protect against hydrogen peroxide-induced cell death [8]. Our study showed that the large isoforms A and B functionally protect against oxidative stress (Fig. 2C).

Intracellular ROS damage mtDNA and trigger further ROS formation in a vicious cycle. This is consistent with our observation that hOXR1 depletion increased the levels of mtDNA damage and hydrogen peroxide-induced oxidative stress, whereas depletion of hOXR1 in cells lacking mtDNA caused no significant difference in ROS formation. Thus, hOXR1 is important for maintaining mitochondrial genome stability by counteracting hydrogen peroxide-induced oxidative stress in the cells (Fig. 6).

Author contributions

The project was designed by M.B., M.Y., and L.E. The experiments were performed by M.Y., J.G.S., R.F.J., and P.H.B. The data were analyzed, discussed, and interpreted by all the authors. The article was written by M.B., M.Y., L.E., and L.L.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2014.09.003>.

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