

Article

# Reduction of Hydrogen Peroxide by Human Mitochondrial Amidoxime Reducing Component Enzymes

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**Abstract:** The mitochondrial amidoxime reducing component (mARC) is a human molybdoenzyme known to catalyze the reduction of various *N*-oxygenated substrates. The physiological function of mARC enzymes, however, remains unknown. In this study, we examine the reduction of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by the human mARC1 and mARC2 enzymes. Furthermore, we demonstrate an increased sensitivity toward H<sub>2</sub>O<sub>2</sub> for HEK-293T cells with an *MTARC1* knockout, which implies a role of mARC enzymes in the cellular response to oxidative stress. H<sub>2</sub>O<sub>2</sub> is a reactive oxygen species (ROS) formed in all living cells involved in many physiological processes. Furthermore, H<sub>2</sub>O<sub>2</sub> constitutes the first mARC substrate without a nitrogen–oxygen bond, implying that mARC enzymes may have a substrate spectrum going beyond the previously examined *N*-oxygenated compounds.

**Keywords:** reactive oxygen species; hydrogen peroxide; molybdenum enzyme



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## 1. Introduction

The human mARC enzyme was first described in 2006 as the third component of the *N*-reducing complex together with hemoprotein cytochrome b5 (Cyb5B) and flavoprotein cytochrome b5 reductase (Cyb5R3) [1]. Together with these two electron carrier proteins, mARC enzymes reduce various *N*-hydroxylated substrates like amidoximes, *N*-hydroxyguanidines, hydroxylamines, *N*-oxides or hydroxamic acids [2]. mARC utilizes a Mo-molybdopterin cofactor (Moco), the coordination of the catalytic molybdenum ion being very similar to that observed in sulfite oxidase (SO) [3,4] despite not sharing many other characteristics with SO. Thus, mARC enzymes are classified as part of a separate, new family of molybdenum enzymes, the MOSC domain family [5,6]. All mammalian genomes encode two paralogues of mARC: mARC1 and mARC2 (gene names *MTARC1*, *MTARC2*) [4].

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is the major reactive oxygen species (ROS) in eukaryotic cells and some 37 human enzymes are known to generate H<sub>2</sub>O<sub>2</sub> by the two-electron reduction of dioxygen [7]. Interestingly, among these H<sub>2</sub>O<sub>2</sub>-producing enzymes are the eukaryotic molybdenum enzymes xanthine oxidase (XO) [8], aldehyde oxidase (AO) [9] and sulfite oxidase (SO) [10].

While high concentrations of H<sub>2</sub>O<sub>2</sub> cause oxidative damage to cells, it is considered to be a physiologically relevant signaling molecule at lower concentrations. These different effects of H<sub>2</sub>O<sub>2</sub> have been reviewed in detail elsewhere [7].

Various cell compartments contain H<sub>2</sub>O<sub>2</sub>-degrading enzymes, ensuring tight regulation of H<sub>2</sub>O<sub>2</sub> concentrations. Examples are catalase or myeloperoxidase, which, due to their high *K<sub>M</sub>* values, are suitable for degrading high H<sub>2</sub>O<sub>2</sub> concentrations, e.g., in

peroxisomes [11,12]. Other enzymes like GPx1 or peroxiredoxins act at much lower  $H_2O_2$  concentrations characteristic for their respective cell compartments [13,14].

Until now, no  $H_2O_2$ -degrading enzyme has been identified in the outer mitochondrial membrane (OMM), where mARC enzymes are localized [15].

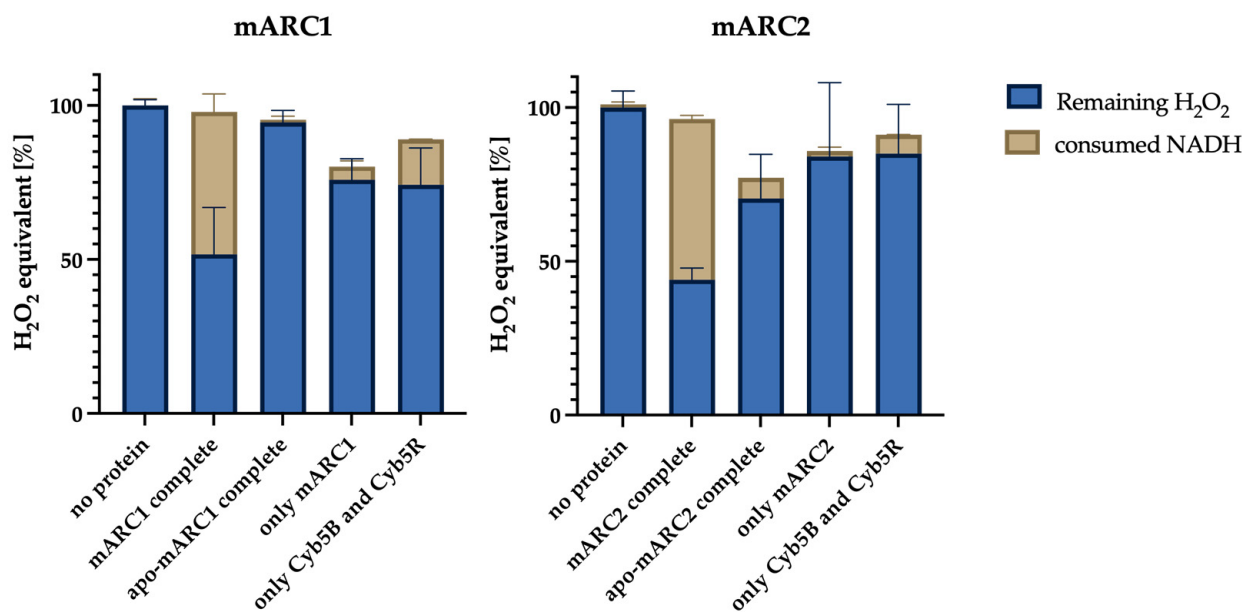
The mARC enzyme system is known best for its reductive activity toward *N*-oxygenated compounds. However, some studies have shown links between mARC and ROS. For example, the common mARC1 p.A165T variant is associated with higher levels of lipid peroxidation, while the total antioxidant activity (TAA) in serum and expression of catalase are increased [16].

In this work, we present the NADH-dependent degradation of  $H_2O_2$  by the human mARC1 and mARC2 enzymes in concert with their electron carriers Cyb5B and CYB5R3 using recombinant proteins. We go on to show the effect of an *MTARC1* knockout on the viability of HEK-293T cells in the presence of high external  $H_2O_2$  concentrations.

## 2. Results

### 2.1. Molybdenum-Containing mARC1 and mARC2 Both Reduce $H_2O_2$

To assess the reduction of  $H_2O_2$  by mARC enzymes, we compared the NADH consumption, measured in the NADH assay, and the amount of residual  $H_2O_2$ , quantified by the fluorometric assay, for several different setups. Importantly, extensive control reactions were examined to unambiguously identify the effect of molybdenum-containing mARC enzymes. The results from these assays are visualized in Figure 1. Note that different  $H_2O_2$  concentrations (50  $\mu$ M for mARC1 and 80  $\mu$ M for mARC2) were used due to different stabilities of the enzymes toward high  $H_2O_2$  concentrations.



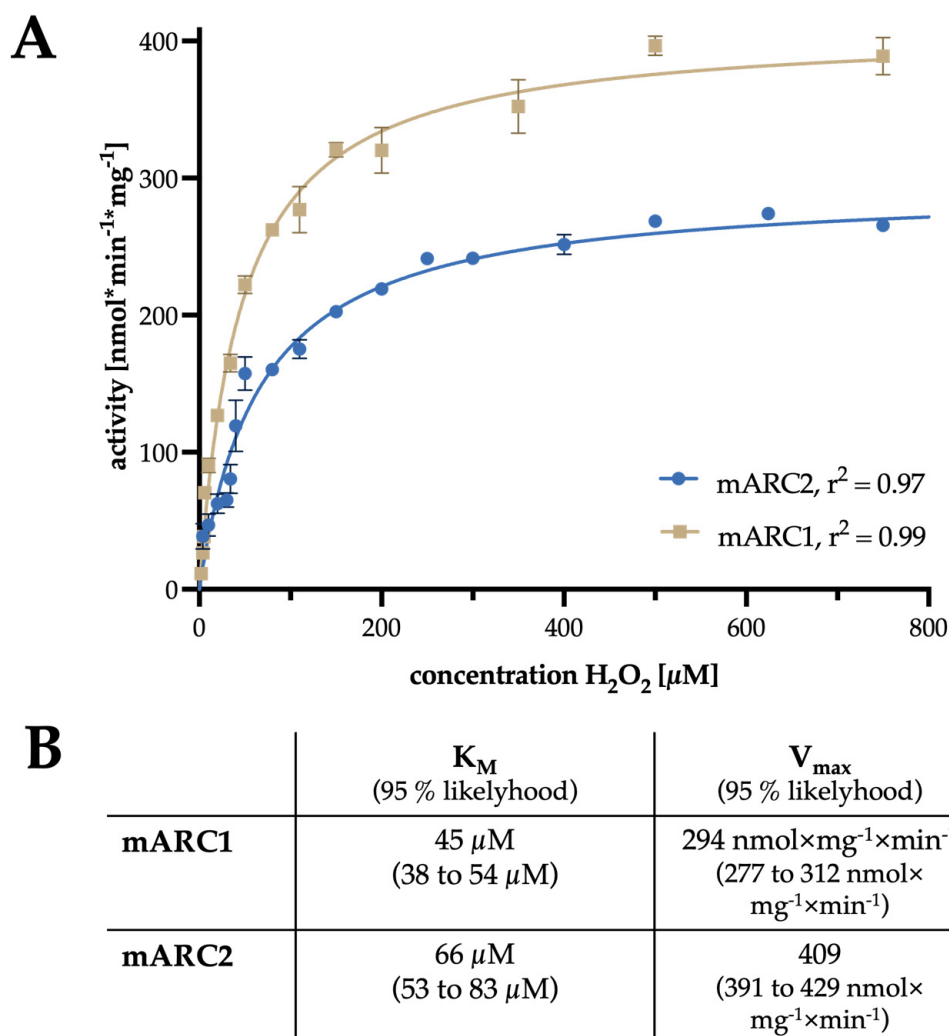
**Figure 1.** Stacked bar-chart representing the consumption of NADH (brown columns) on top of the residual amount of  $H_2O_2$  (blue columns) for mARC1 (panel A) and mARC2 (panel B). The individual setups compared to each other are (from left to right): no protein—assay containing only NADH and  $H_2O_2$  but no enzymes; mARC1/2 complete—contains mARC1 or mARC2 and both electron carrier proteins; apo-mARC1 complete—same as before but with molybdenum-free apo-mARC; only mARC1/2—just mARC1/2, but no electron carriers; only Cyb5B and Cyb5R—only electron carriers but no mARC enzymes.

It is clearly observed that, for both mARC1 and mARC2, the by far greatest depletion of NADH is observed when the complete, reconstituted mARC1/2 enzyme systems are used. Correspondingly, in these reactions, the lowest concentrations of residual  $H_2O_2$  were found, which confirms that NADH consumed by the mARC enzyme system does

in fact reduce  $\text{H}_2\text{O}_2$ . The control reactions indicate that only holo-mARC enzymes with a molybdopterin prosthetic group can reduce  $\text{H}_2\text{O}_2$  in concert with Cyb5B and Cyb5R3.

### 2.2. Kinetics of mARC-Dependent $\text{H}_2\text{O}_2$ Reduction

Both mARC1 and mARC2 display Michaelis–Menten kinetics for  $\text{H}_2\text{O}_2$  reduction, as is shown in Figure 2. The turnover rates and  $K_M$  values for  $\text{H}_2\text{O}_2$  reduction by mARC1 and mARC2 are comparable, with mARC1 showing a slightly lower  $K_M$  but higher turnover rates.



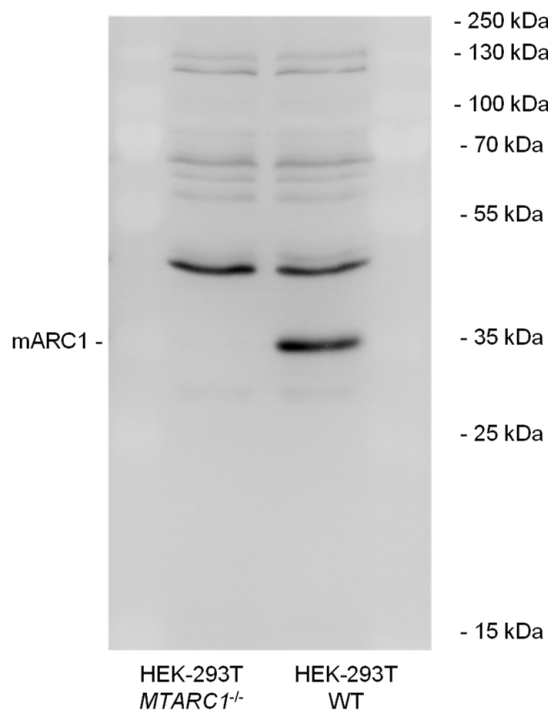
**Figure 2.** (A) Kinetic profile of mARC1- and mARC2-catalyzed  $\text{H}_2\text{O}_2$  reduction. (B) Kinetic parameters obtained from fitting to the Michaelis–Menten equation. Values in parentheses indicate the 95% likelihood interval for  $K_M$  and  $V_{max}$ .

Using our recently established fluorescence-based high-throughput assay [17], we were able to measure very similar conversion rates for the mARC-catalyzed reduction of hydrogen peroxide.

### 2.3. MTARC1 Knockout Decreases Cell Viability in Presence of $\text{H}_2\text{O}_2$

To determine whether or not the reduction of  $\text{H}_2\text{O}_2$  by recombinant mARC enzymes is relevant in cellulo, we examined the impact of different  $\text{H}_2\text{O}_2$  concentrations on cell viability using an HEK-293T-based knockout model. Since HEK-293T cells express only very low levels of mARC2, the *MTARC1* knockout results in cells practically devoid of mARC activity (mARC2 expression levels do not increase to compensate the *MTARC1*

knockout. The knockout was shown to be effective on the protein level by Western blot analysis (Figure 3). Before incubation with H<sub>2</sub>O<sub>2</sub>, some cells were treated with buthionine sulfoximine (BSO), an inhibitor of glutathione synthesis.

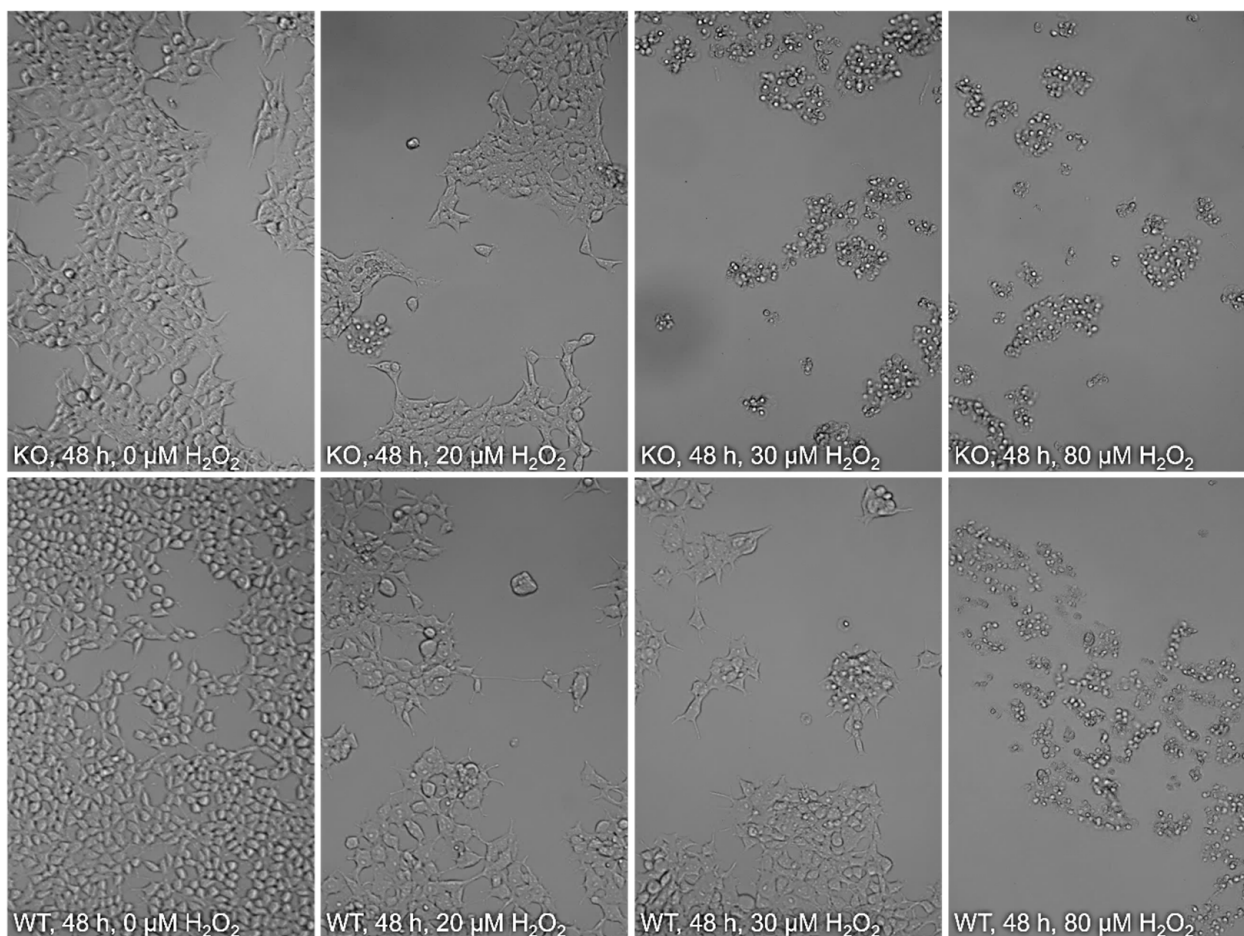


**Figure 3.** Protein production to verify *MTARC1* KO. *MTARC1*<sup>-/-</sup> and WT cells were lysed, 36 µg of protein was applied per lane and Western blot analyses were performed using an anti-mARC1 antibody.

Differences seen between wildtype and knockout cells are already reflected by cell morphology. A changed cell morphology induced by H<sub>2</sub>O<sub>2</sub>, which can be observed in KO cells at 20 µM, only occurs in WT cells at 30 µM, while the KO cells at 30 µM can hardly be considered morphologically alive (Figure 4). Furthermore, Hoechst staining revealed that knockout cells treated with 30 µM H<sub>2</sub>O<sub>2</sub> had higher nuclear condensation and thereby an increased apoptosis rate compared to wildtype cells (Figure 5C).

This observation was confirmed in a resazurin-based cell viability assay. While low concentrations of H<sub>2</sub>O<sub>2</sub> do not appear to have a negative influence on cell viability in either WT or KO cells, when increasing H<sub>2</sub>O<sub>2</sub> concentrations above 10 µM, WT and KO cells clearly show divergence, with the viability of WT cells being significantly higher (Figure 5A,B). A decreased viability of knockout cells can already be observed after 8 h and only becomes even more pronounced after longer incubation periods. After 48 h, KO cells are not viable at 30 µM H<sub>2</sub>O<sub>2</sub>, whereas the same is observed with WT cells at a concentration of 80 µM H<sub>2</sub>O<sub>2</sub>. At a concentration of 30 µM, the viability of the WT cells is still approx. 70%.

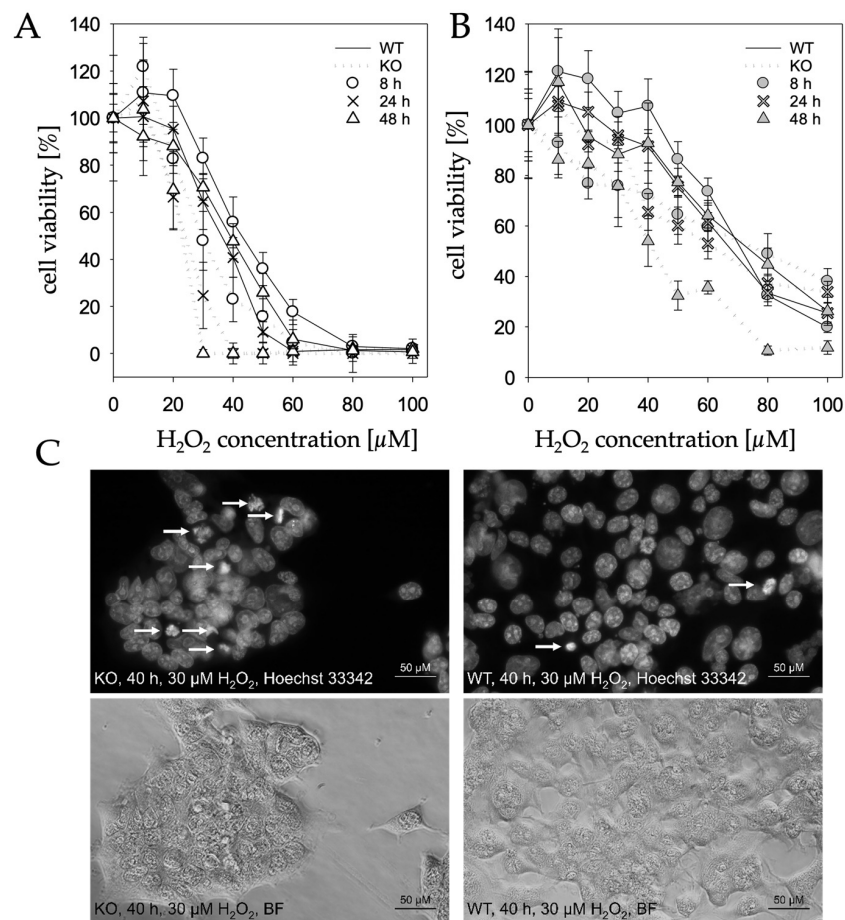
These findings show that H<sub>2</sub>O<sub>2</sub> degradation by the mARC1 enzyme does occur in cell culture, and it has a measurable effect on cell physiology at high extracellular H<sub>2</sub>O<sub>2</sub> concentrations.



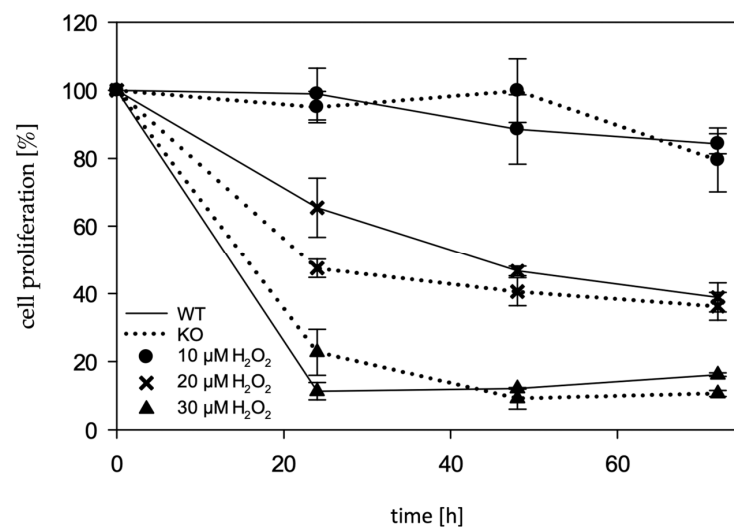
**Figure 4.** Cytotoxicity of  $\text{H}_2\text{O}_2$  to HEK-293T *MTARC1*-KO and WT cells. Cells were seeded onto 96-well plates and incubated with medium containing 0.3 mM BSO for 16 h, followed by incubation with medium containing 20–80  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . After 48 h of incubation, cell morphology was examined microscopically. At 0–20  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , both WT and KO cells have very similar morphologies. However, when the  $\text{H}_2\text{O}_2$  concentration is increased to 30  $\mu\text{M}$ , morphology of KO cells changes drastically, whereas the WT cells look largely unaffected. At 80  $\mu\text{M}$ , both cell lines display strong morphological differences, resembling the changes already seen at 30  $\mu\text{M}$  for KO cells.

Further, an influence on cell proliferation could be observed. Extracellular concentrations of 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  showed no impairment on cell proliferation. An extracellular  $\text{H}_2\text{O}_2$  concentration of 20  $\mu\text{M}$  did lead to impaired cell proliferation: after 24 h, the proliferation of both WT and KO cells was decreased compared to control cells without  $\text{H}_2\text{O}_2$  treatment. This impairment on cell proliferation was more pronounced in *mARC1*-deficient cells, where, after 24 h, only 50% could be counted compared to cells treated with the medium only; thus, in purely arithmetical terms, no cell division of the KO cells had taken place in the last 24 h. The number of WT cells was reduced to approx. 70%. After 72 h, both WT and KO cells were reduced to approx. 35%. At a  $\text{H}_2\text{O}_2$  concentration of 30  $\mu\text{M}$ , no measurable cell division occurred in either the KO or WT cells (Figure 6).





**Figure 5.** Cell viability and Hoechst 33342 staining. Cells were treated with different concentrations of H<sub>2</sub>O<sub>2</sub> and examined after 8 h, 24 h, 48 h and 72 h by resazurin assay. Cells treated with medium without supplemented H<sub>2</sub>O<sub>2</sub> were defined as 100% viability. (A) Cell viability after prior treatment with 0.3 mM BSO; (B) cell viability without prior treatment with BSO; (C) fluorescence and bright film microscopy of HEK-293T *MTAR1* KO and WT cells in a 40× objective magnification. Cells were treated for 48 h with 30 μM H<sub>2</sub>O<sub>2</sub>. The white arrows mark cell nuclei with clear chromatin condensation.



**Figure 6.** Cell proliferation. Cells were treated with different concentrations (10 μM, 20 μM, 30 μM) of H<sub>2</sub>O<sub>2</sub>. After 24 h, 48 h and 72 h, the cell number was determined on the ImageXpress<sup>®</sup> (λ<sub>ex</sub>: 358, λ<sub>em</sub>: 461) (Molecular Devices, LLC., San Jose, CA, USA).

### 3. Discussion

The study presented here identifies  $H_2O_2$  as a new substrate for the human mARC1 and mARC2 proteins. The degradation of  $H_2O_2$  was demonstrated in vitro with recombinant enzymes and confirmed in a more complex environment by in cellulo knockout studies. Thus, for the first time, a reduction of O-O bonds by the mARC enzyme system is described. Molybdenum enzymes like mARC typically characterize two-electron transfer reactions; therefore, the product of this reaction is likely water.

While we cannot at this point conclude that  $H_2O_2$  or other reactive oxygen species are the physiological substrate of eukaryotic mARC enzymes, this finding is important nonetheless. Compounds with O-O bonds are a completely novel group of potential mARC substrates that have not previously been studied. So far, all mARC-catalyzed reactions described in the literature are N-reductions cleaving N-O bonds [18].

The turnover rates of the mARC-catalyzed  $H_2O_2$  reduction are relatively low compared to other  $H_2O_2$ -degrading enzymes. It should be noted, however, that turnover rates determined with the soluble recombinant proteins without their OMM-anchoring sequences are typically much lower for human mARC enzymes compared with proteins isolated from organ homogenates [19]. Thus, in vivo  $H_2O_2$ -reducing activities of human mARC enzymes can be expected to be significantly higher than the values reported here. On another note, the  $K_M$  values for  $H_2O_2$  reduction by recombinant human mARC proteins (approx. 50  $\mu M$ ) are very low compared to those for the well-studied N-hydroxylated compounds.

Hydrogen peroxide has fundamentally important functions in humans. Depending on the intracellular concentration, it initiates, inter alia, cell proliferation, cell shaping, migration and angiogenesis [20–22]. On the other hand, the accumulation of higher concentrations of hydrogen peroxide and other ROS leads to oxidative stress, a condition of imbalance between pro-oxidants and antioxidants. ROS pass through cell membranes and cause oxidative damage to lipids, proteins and DNA, as well as mitochondrial dysfunction, all of which can lead to the loss of essential cell functions and initiate the caspase-mediated apoptosis pathway [23–25].

In this study, a coupled enzyme assay was established. Two parameters were measured: the amount of NADH oxidized by the mARC-mediated reduction and, to confirm the results, the remaining concentration of hydrogen peroxide. Thus, it was shown that both mARC proteins can reduce  $H_2O_2$ .

Considering the enzyme kinetics of this reaction, it is striking that the  $K_M$  values of both mARC proteins are remarkably lower when compared to well-known  $H_2O_2$ -depleting enzymes such as catalase or peroxiredoxin [26,27].

An in cellulo *MTARC1* knockout model was generated and established to verify whether the absence of mARC1 leads to cellular impairment upon exposure to  $H_2O_2$ . A significantly reduced cell physiology of mARC1-deficient cells and thus a higher sensitivity toward  $H_2O_2$  compared to corresponding WT cells could be observed. Also, higher apoptosis levels and lower cell viability levels were seen. These findings could be confirmed by light and fluorescence microscopy showing altered cell morphology and declined nuclear condensation. While the  $H_2O_2$  concentrations used in our cell culture experiments certainly exceed those expected in vivo, it is still possible that the regulation of ROS is a physiological function of mARC enzymes.

The complex mechanisms of hydrogen peroxide regulation with a large number of enzymes in various cell organelles demonstrate the need for different approaches to control the intracellular concentration. While major hydrogen peroxide transforming enzymes like catalase, GPx or peroxiredoxins are present in the endoplasmic reticulum, cytosol, nucleus, peroxisomes, the intermembrane space (IMS), inner mitochondrial membrane (IMM) and mitochondrial matrix, mARC stands out through its localization at the OMM [13,15,28–30], although there are some reports about GPx also being localized at the OMM [31].

mARC might thus be involved in protecting the OMM from ROS. Since ROS are formed in high concentrations in the IMS and the cytosol by different enzymes, a protective mechanism for the undesired oxygenation of the OMM—for example, against lipid

peroxidation to prevent oxidative stress and mitochondrial dysfunction—is conceivable. This was also described earlier for GPx-4 at the IMM [28]. There are also some known enzymes at the OMM, such as the monoamine oxidase MAO, that form hydrogen peroxide as a secondary product [28]. It is thus also possible that mARC influences the free diffusion between IMS and cytosol diffusion and the transport of H<sub>2</sub>O<sub>2</sub> through voltage-dependent anion channels (VDACs) and peroxiporins due to its high affinity to H<sub>2</sub>O<sub>2</sub>.

Kagan and colleagues highlighted the significance of ROS for apoptosis by identifying the releasing pathway of proapoptotic factors from the OMM. A H<sub>2</sub>O<sub>2</sub>-dependent cardiolipin-specific peroxidase activity of cytochrome c is required for the permeabilization of the OMM, demonstrating again the significance of hydrogen peroxide regulation in the OMM for critical cell processes [32]. Also, H<sub>2</sub>O<sub>2</sub> is formed in the peroxisomal  $\beta$ -oxidation of fatty acids [33]. Various studies in mice and rats also suggest the possibility of a dual localization of mARC in mitochondria and peroxisomes [34,35]; thus, this colocalization could suggest that mARC has a regulatory function in hydrogen peroxide and antioxidant metabolism in peroxisomes as well.

In conclusion, the reduction of H<sub>2</sub>O<sub>2</sub> by mARC is certainly very interesting, as it indicates that the spectrum of substrates that these enzymes can reduce could go far beyond the previously studied *N*-oxygenated substrates. The in cellulo studies confirm that H<sub>2</sub>O<sub>2</sub> is also reduced by the native mARC enzyme in its cellular context. We do not claim that H<sub>2</sub>O<sub>2</sub> or other reactive oxygen species are the physiological substrates of mARC. An involvement in the cellular regulation of H<sub>2</sub>O<sub>2</sub> is conceivable, but data available on this point are not sufficient to claim this to be the enzymes' function. Due to their involvement in liver disease, mARC enzymes have recently gained much attention. However, it remains unknown what the physiological function of mARC actually is and how exactly it exerts its influence on lipid metabolism and liver disease. In the future, a search for mARC's physiological substrate that might not have previously been associated with mARC should be considered.

## 4. Materials and Methods

### 4.1. Protein Sources

Recombinant human mARC1, mARC2, Cyb5B and Cyb5R3 were expressed in *Escherichia coli* (*E. coli*) and purified by column chromatography, essentially as described previously [36]. For mARC1 and mARC2 with bound molybdopterin cofactor (*holo*-mARC1/2), the *E. coli* TP1000 strain [37] was used. Proteins without molybdopterin were expressed in RK5202 [38]. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin for calibration. Loading of Cyb5B with heme and Cyb5R3 with flavin adenine dinucleotide (FAD) was quantified as published [36].

### 4.2. Photometric Assay

Reduction of H<sub>2</sub>O<sub>2</sub> by the reconstituted mARC enzyme system was assayed using the previously published protocol [2]. Reactions contained 7.5  $\mu$ g (224 pmol) of either mARC1 or mARC2, 3.5  $\mu$ g (210 pmol) Cyb5B and 0.08  $\mu$ g (2.4 pmol) Cyb5R3 and 200  $\mu$ M NADH in 20 mM Na-MES buffer, pH 6.0. The total reaction volume was 300  $\mu$ L. Consumption of NADH at 37 °C was monitored by recording the absorption spectrum from 300 to 400 nm in 15 s intervals. The reaction was stopped by heating 200  $\mu$ L of the incubation mix to 95 °C for 5 min in a water bath. Turnover rates were calculated through the change in absorption at 340 nm over a timespan of 2 min. Kinetic parameters were determined by fitting the Michaelis–Menten equation to the turnover rates at different H<sub>2</sub>O<sub>2</sub> concentrations in GraphPad Prism 9.5.1. All measurements were performed in triplicate.

### 4.3. Fluorometric Activity Assay

Alternatively, the enzyme activity was assayed by monitoring NADH consumption through a recently established fluorometric protocol [17]. Briefly, time-dependent change



in NADH fluorescence ( $\lambda_{\text{ex}} = 340 \text{ nm}$ ;  $\lambda_{\text{em}} = 365 \text{ nm}$ ) was monitored with a TECAN Infinite 200 M Pro plate reader. The reaction volume was 50  $\mu\text{L}$ . Assays contained 193 pmol (=6.5  $\mu\text{g}$ ) *hmARC-1*, 65 pmol *hCyb5B* (heme), 6.5 pmol *hCyb5R3* (FAD), 0.2 mM of NADH and the substrate to be tested in 20 mM Na-MES buffer, pH 6.0. The reaction mixtures containing all components except *Cyb5R3* were pre-incubated at 37 °C for 3 min. The reactions were started by adding *Cyb5R3*, and NADH fluorescence was recorded for 15 min at 37 °C. BAO was always used in parallel as a reference substrate.

#### 4.4. Peroxide Assay

To confirm degradation of  $\text{H}_2\text{O}_2$  by the mARC enzyme system, residual  $\text{H}_2\text{O}_2$  concentrations were quantified using a fluorometric peroxidase assay [39]. Samples from the photometric activity assays were cooled on ice for 1 min and then pre-incubated at 37 °C for 2 min. Then, 10  $\mu\text{L}$  of 20 mM Na-MES buffer, pH 6.0, 30  $\mu\text{L}$  of 20 mM 4-hydroxyphenylacetic acid and 30  $\mu\text{L}$  of a 30  $\mu\text{g}/\text{mL}$  horseradish peroxidase solution were added and incubation at 37 °C was continued for 10 min. Afterwards, 10  $\mu\text{L}$  of 10 M NaOH and 850  $\mu\text{L}$  distilled water were added. A total of 150  $\mu\text{L}$  was transferred to Perkin Elmer quartz SUPRASIL cuvettes. Fluorescence spectra from 340 to 450 nm were measured in a Perkin Elmer LS 55 Fluorescence Spectrometer using an excitation wavelength of 320 nm. The peak at 408 nm was used for evaluation. Correlation between the intensity of this peak and the  $\text{H}_2\text{O}_2$  concentration was proven using a calibration curve.

#### 4.5. Molecular Biology

Knockout of the *MTARC1* gene in HEK-293T cells was achieved by the CRISPR-Cas9 method [40]. A sequence encoding sgRNA for sgRNA addressing exon 2 of the *MTARC1* gene (5'-GTGGCCAAAACCGAACACTAGT-TGG-3', PAM sequence underlined) was cloned into the Esp3I site of the plentiCRISPRv2 plasmid (Addgene #49535) using standard cloning methods [41]. Correct insertion of the sgRNA-encoding sequence was confirmed by Sanger sequencing using the primer 5'-GAGGGCCTA-TTCCCATGATTCC-3'.

#### 4.6. Mammalian Cell Culture

Human embryonic kidney cells (HEK-293T) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a humidified incubator at 37 °C in presence of 5%  $\text{CO}_2$ . HEK-293T cells were verified by SNP analysis and confirmed to be mycoplasma-free.

For transfection, target cells were seeded at  $2 \times 10^5$  cells/well in a 6-well plate. Twenty-four hours after seeding, the cultivation medium was replaced with DMEM containing 2% FCS. The transfection mix consisted of 100  $\mu\text{L}$  Opti-MEM, 1  $\mu\text{g}$  DNA (plentiCRISPRv2 containing the sgRNA sequence) and 3  $\mu\text{L}$  Lipofectamine 2000<sup>®</sup> (Thermo Fisher Scientific, Waltham, MA, USA). Medium was exchanged for DMEM incl. 10% FCS after 6 h. After further 18 h, medium was replaced again by DMEM incl. 10% FCS, supplemented with 2.5  $\mu\text{g}/\text{mL}$  puromycin. Cells were cultivated and selected in puromycin-containing medium for 6 days.

HEK-293T KO lines were isolated by serial dilution in 96-well plates (0.5 cells/well). After three weeks of expansion, DNA was isolated with the peqGOLD microspin tissue DNA kit (VWR, Darmstadt, Germany), the region of interest was amplified by PCR and the knockout was validated by Sanger sequencing. Primers for both amplification of the gene region of interest as well as sequencing were 5'-AAGCTCCTCCAGGGTCTGGCTTC-3' and reverse 5'-CGACCTGCCCTTTCCTTACCTGC-3'.

For immunoblot analysis, cells were detached using ice-cold Dulbecco's PBS (DPBS), centrifuged, and resuspended in NP-40 lysis buffer (containing 150 mM NaCl, 1% (*v/v*) Nonidet P-40, 50 mM Tris). After 30 min shaking at 4 °C, the lysate was centrifuged again and the protein concentration in the supernatant was quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

#### 4.7. SDS-PAGE and Immunoblotting

Samples containing 36 µg of total protein were separated by SDS-PAGE on hand-cast MiniProtean gels supplemented with 0.5% trichloroethanol (TCE) (*v/v*) (Bio-Rad, Hercules, CA, USA) according to standard protocols. TCE was used as an unspecific protein staining. It reacts with tryptophan residues of the proteins under UV radiation for 5 min to a fluorescent product [42]. After electrophoresis, proteins were transferred onto Hybond-P polyvinylidene fluoride membranes (GE Healthcare, Chicago, IL, USA). The membranes were blocked in TRIS-buffered saline containing 0.1% Tween 20 (TBST) and 5% milk powder, incubated with primary antibodies and washed with TBST. Antibodies used were anti-mARC1 (Abgent, San Diego, CA, USA; AP9754c, 1:1000 dilution) and a horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Ely, UK). Fluorescence and chemiluminescence were detected on a ChemoStar Touch ECL and Fluorescence Imager (Intas Science Imaging, Göttingen, Germany).

#### 4.8. Viability Assay

Both wildtype and *MTARC1* knockout HEK-293T cells were seeded at 3000 cells per well into 96-well plates containing 80 µL DMEM (with 10% FCS). Twenty-four hours after seeding, adherent cells were incubated with medium containing 0.3 mM BSO for 16 h, followed by incubation with medium containing 0, 10, 20, 30, 40, 50, 60, 80, 100 µM H<sub>2</sub>O<sub>2</sub>. Cell viability was assayed using a water-soluble resazurin assay (Sigma Aldrich) after 8, 24 and 48 h. A total of 11 µL of a 0.01% resazurin solution in PBS was added directly to the culture medium (10% of the culture medium volume, 0.001% resazurin). After 180 min incubation at 37 °C, the amount of converted resazurin was measured fluorometrically ( $\lambda_{\text{ex}} = 530 \text{ nm}$ ,  $\lambda_{\text{em}} = 590 \text{ nm}$ ) in a spark<sup>®</sup> multimode microplate reader (Tecan Trading AG, Männedorf, Switzerland). The fluorescence measured for cells treated with 0 µM H<sub>2</sub>O<sub>2</sub> were defined as 100% viability. mARC1 itself does not significantly contribute to resazurin reduction.

#### 4.9. Proliferation Assay

To determine the influence of H<sub>2</sub>O<sub>2</sub> on cell proliferation, the same number of cells were seeded in black 96-well microtiter plates with transparent bottom. Cells were fixed and stained with Hoechst 33342 after further 24 h, 48 h and 72 h of incubation with 10 µM, 20 µM and 30 µM H<sub>2</sub>O<sub>2</sub>. For this purpose, 50 µL of 100 µL culture medium was removed and replaced by 50 µL of an 8% PFA, 0.002% Hoechst 33342 solution in DPBS. After 10 min of incubation at RT, the supernatant was completely removed, and each well was washed twice with DPBS. Cells were overcoated with DPBS and counted on the ImageXpress<sup>®</sup> ( $\lambda_{\text{ex}}: 358$ ,  $\lambda_{\text{em}}: 461$ ) (Molecular Devices, LLC., San Jose, CA, USA).

#### 4.10. Microscopy

After 40 h of incubation at 37 °C, cells were imaged live at 20× objective magnification on an Olympus CK2 microscope. For Hoechst 33342 staining, 1500 cells were seeded onto 96-well half-area black microplates and incubated with H<sub>2</sub>O<sub>2</sub> as described before. Cells were fixed with 8% formaline in PBS for 10 min at room temperature and simultaneously stained with Hoechst 33342. After staining, wells were rinsed twice with PBS to remove any remaining dye. Apoptotic cells were observed under a fluorescence microscope at 40× objective magnification (Olympus, Tokyo, Japan).

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