[**Review**]

Peroxiredoxin 4 (PRDX4): Its Critical *In Vivo* Roles in Animal Models of Metabolic Syndrome Using Our Unique PRDX4 Transgenic Mice

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Abstract

The peroxiredoxin (PRDX) family, a new family of proteins with a pivotal antioxidative function, is ubiquitously synthesized and abundantly identified in various organisms. In contrast to the intracellular localization of other family members (PRDX1/2/3/5/6), PRDX4 is the only known secretory form and protects against oxidative damage by scavenging reactive oxygen species in both the intracellular (especially the endoplasmic reticulum) compartments and the extracellular space. Recently, we generated unique human PRDX4 (hPRDX4) transgenic (Tg) mice on a C57BL/6J background and investigated the critical and diverse protective roles of PRDX4 against diabetes mellitus, atherosclerosis, insulin resistance, and nonalcoholic fatty liver disease (NAFLD) as well as evaluated its role in the intestinal function in various animal models. Our published data have shown that PRDX4 helps prevent the progression of metabolic syndrome by reducing local and systemic oxidative stress and synergistically suppressing steatosis, inflammatory reactions, and/or apoptotic activity. These observations suggest that Tg mice may be a useful animal model

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for studying the relevance of oxidative stress on inflammation and the dysregulation of lipid/bile acid/glucose metabolism upon the progression of human metabolic syndrome, and that specific accelerators of PRDX4 may be useful as therapeutic agents for ameliorating various chronic inflammatory diseases.

Key words: Peroxiredoxin (PRDX) 4, chronic inflammatory disease, metabolic syndrome, human PRDX4 transgenic mice (Tg), animal model.

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Introduction

Accumulating evidence has revealed that oxidative stress and endoplasmic reticulum (ER) stress, including oxidized molecules and reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and the concomitant generation of nitric oxide (NO), strongly contribute to the initiation and progression of chronic inflammatory diseases, such as diabetes mellitus (DM) or atherosclerosis [1–4]. Oxygen (O_2), necessary for the vital activities of living organisms, is paradoxically a highly reactive molecule and can easily form the one-electron reduction state of O_2^-, subsequently resulting in the formation of H_2O_2, as shown in Figure 1. Increased oxidative stressors within the extracellular space are considered a risk factor of atherosclerosis due to the accumulation of oxidized low-density lipoprotein (oxLDL) as well as the formation of ROS [4–6], a feature of chronic inflammatory processes. The vascular vessels range from small to large sizes, with atherosclerotic lesions histopathologically termed “arteriolosclerosis” to “atheroma” [7–9]. DM is closely related to the pathophysiological aspects of

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arteriolosclerosis, and metabolic syndrome, manifesting as obesity, dyslipidemia, type 2 DM and/or atherosclerosis, also has a tight correlation with the development of nonalcoholic fatty liver disease (NAFLD) [8–11], which is an umbrella term covering a broad spectrum of clinicopathological presentations, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) [10–18]. It is well known that NASH is not only a more severe form of NAFLD, occasionally leading to end-stage liver diseases, including cirrhosis and/or hepatocellular carcinoma (HCC) [12–18], but also that oxidative stress and ER stress appear to be critically involved in these diseases' processes, even in animal models [16–21]. In fact, oxidative stress appears, at least in part, to mechanistically illustrate the endothelial dysfunction, and the vascular inflammation and complications especially in the initiation of the metabolic syndrome/metabolic syndrome-related diseases [1,2,5–11]. Furthermore, in addition to the target organs of metabolic syndrome (arteries and liver), the small intestine with its extensive surface area is the first organ to encounter nutrients, which likely plays an important role in the development of metabolic disorders as

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well [22,23].

Taken together, these previous findings and our *in vivo* data indicate that metabolic syndrome is an extremely complex disease orchestrated by multiple molecular and histopathologic factors, including not only oxidative stress but also elevated levels of inflammatory cytokines and apoptotic activity and the translocation of either intestinal bacteria or microbial cell components [8–11,24–40]. All researchers should be aware of the fact that lipid/bile acid (BA)/glucose metabolism with both local (each tissue) and whole-body functions plays a central, key role in the etiology of metabolic syndrome.

Peroxiredoxin (PRDX), a new family of antioxidant enzymes, is ubiquitously synthesized and has been abundantly identified in various organisms [41–44]. At least six distinct PRDX genes expressed in mammals possess high similarities in their molecular structures and contain a reactive cysteine (Cys) in a conserved region near the N-terminus that forms Cys-sulfenic acid as an intermediate reaction during the reduction of H_2O_2 [44–46]. As shown in Figure 1, PRDXs regulate the H_2O_2 levels using thioredoxin (TRX) as

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an electron donor, and their functional peroxidase activity is dependent on the reduced forms of TRX (redTRX) and/or glutathione (GSH) [41–46]. Figure 1 summarizes the pathway of the detoxification of ROS by PRDXs. Within the PRDX family, PRDX4 is a member of the 2-Cys-type PRDX family and homologous to typical 2-Cys PRDX1 and 2, thus serving as a TRX-dependent peroxidase via a similar catalytic mechanism to PRDX1/2 [44,47]. On one hand, since PRDX4 could be highly susceptible to hyperoxidation/inactivation due to H_2O_2-mediated sulfonic acid formation on its peroxidatic Cys [41–47], PRDX4 might be a weak antioxidative enzyme compared to other thiol-dependent antioxidants in the context of metabolic syndrome-related diseases. On the other hand, PRDX4 is the only known secretory form located in not only the intracellular (especially the ER) but also the extracellular space, in contrast to the intracellular localization of other family members (PRDX1, 2 and 6 are located in the cytoplasm, and PRDX3 and 5 in mitochondria) [41–44,48,49]. PRDX4 has an additional N-terminal region, which is unique to this enzyme, following a signal peptide that allows translocation across

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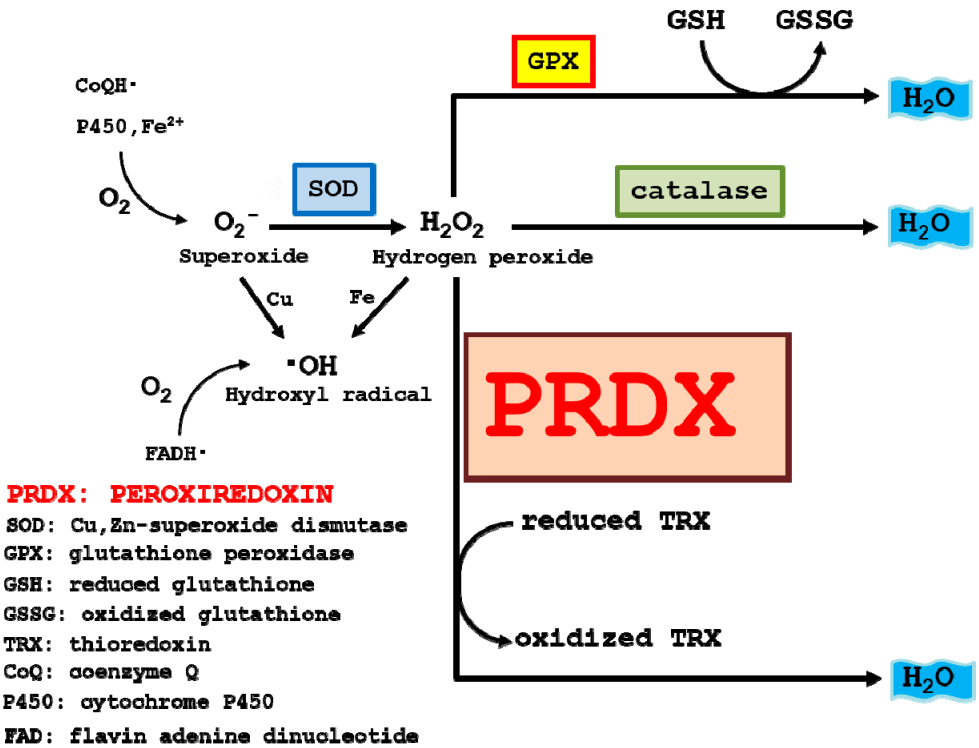
the ER membrane and is thereafter cleaved [41–44]. Several previous studies have focused on the molecular regulatory roles of PRDX4 in the nuclear factor κB (NF-κB) [50], epidermal growth factor, and p53 [51] or thromboxane A2 receptor [49] cascade. More recently, PRDX4 has been reported to play a pivotal role in the disulfide bond formation (i.e. oxidative folding) of lipoproteins in coordination with protein disulfide isomerase (PDI) and ER oxidoreductin 1 (ERO1) in the ER [52,53]. However, the detailed *in vivo* pathological and physiological relevance of PRDX4 has remained unclear.

Figure 1



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We hypothesized that PRDX4 locally (intracellularly) and systemically (extracellularly) plays a critical, diverse role *in vivo* in the protection against the initiation and development of metabolic syndrome manifesting as visceral obesity, DM, atherosclerosis and/or NAFLD (i.e. disordered lipid/BA/glucose metabolism). Recently, we newly generated human PRDX4 (hPRDX4) transgenic (Tg) mice and evaluated the *in vivo* functions of PRDX4 in serial studies of various animal models [7–11], as summarized in Table 1. In the current review, we focus on the crucial *in vivo* roles of PRDX4 in various chronic inflammatory diseases, with particular focus on DM, atherosclerosis, and NAFLD. Our findings here suggest that our Tg mice may be a useful animal model for studying the relevance of oxidative stress in ROS- and/or chronic inflammation-induced human diseases, such as metabolic syndrome, and that PRDX4 may be a key factor involved in the pathogenesis of metabolic syndrome.

Table 1. Summary of the *in vivo* roles of PRDX4 in mouse models of metabolic syndrome

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Model	hPRDX4 Tg Mice	References
SHDS-induced type 1 DM	Oxidative stress (ROS)↓ Hyperglycemia↓ Hypoinsulinemia↓ Glucose tolerance↑ Insultis↑ Pancreatic islet areas↑ β-cell apoptosis↓ β-cell proliferation↑ Inflammatory cells/cytokines↓	Ding *et al.*, 2010 [7] Yamada *et al.*, 2012 [8]
HcD-induced Atherosclerosis	Oxidative stress (ROS)↓ Atheroma↓(Anti-atherogenic) Mϕ, SMCs and ECs apoptosis↓ SMC migration/replication↑ Inflammatory cells/cytokines↓ Plaque stability↑ (Mϕ↓, SMC↑, lipid core↓, collagen↑, fibrous cap↑)	Guo *et al.*, 2012 [9]
HFrD+STZ-induced type 2 DM and NAFLD	Oxidative stress (ROS)↓↓ Insulin resistance↓ Glucose tolerance↑ NAFLD↓↓ (steatosis↓, inflammation↓, fibrosis↓) Hepatocyte apoptosis↓ Stellate cell activation↓	Nabeshima *et al.*, 2013 [10]
MCD+HF-induced NAFLD	Oxidative stress (ROS)↓ NAFLD↓ Total intestinal length→∼↑ Villi height→∼↑ Intestinal lipid accumulation↑ Enterocytes apoptosis↓ Enterocytes proliferation↑ Intestinal inflammation↓	Nawata *et al.*, 2016 [11]

Animal models of metabolic syndrome using our unique Tg mice

Construction of Human PRDX4 (hPRDX4) Transgenic (Tg) Mice

Tg mice were generated as described elsewhere [7–11]. The primers for *hPRDX4* are designed based on a published sequence (Genebank accession no. NM_006406). *hPRDX4* cDNA (atggagg cgctgccgct gctagccgcg acaactccgg accacggccg ccaccgaagg ctgcttctgc tgccgctact gctgttcctg ctgccggctg gagctgtgca gggctgggag acagaggaga ggccccggac tcgcgaagag

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gagtgccact tctacgcggg tggacaagtg tacccgggag aggcatcccg ggtatcggtc gccgaccact ccctgcacct aagcaaagcg aagatttcca agccagcgcc ctactgggaa ggaacagctg tgatcgatgg agaatttaag gagctgaagt taactgatta tcgtgggaaa tacttggttt tcttcttcta cccacttgat ttcacatttg tgtgtccaac tgaaattatc gcttttggcg acagacttga agaattcaga tctataaata ctgaagtggt agcatgctct gttgattcac agtttaccca tttggcctgg attaataccc ctcgaagaca aggaggactt gggccaataa ggattccact tctttcagat ttgacccatc agatctcaaa ggactatggt gtatacctag aggactcagg ccacactctt agaggtctct tcattattga tgacaaagga atcctaagac aaattactct gaatgatctt cctgtgggta gatcagtgga tgagacacta cgtttggttc aagcattcca gtacactgac aaacacggag aagtctgccc tgctggctgg aaacctggta gtgaaacaat aatcccagat ccagctggaa agctgaagta tttcgataaa ctgaattga) is amplified by reverse transcription–polymerase chain reaction (RT-PCR) and cloned into the pGEM-T easy vector system (Invitrogen, Life Technologies Japan, Ltd., Tokyo, Japan) [7–11]. The *Not*I fragment containing *hPRDX4* cDNA is inserted into the *Not*I site of pcDNA3 (5.4-kb; Invitrogen, Life Technologies Japan, Ltd.), and a bovine growth hormone polyadenylation (BGHPA) sequence is inserted into the tail of the transgene to stabilize the expression. The entire nucleic acid sequence, containing a 0.6-kb

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cytomegalovirus (CMV) enhancer/promoter, the 0.8-kb *hPRDX4* cDNA, and the 0.2-kb BGHPA sequence, is purified by restriction enzyme digestion with *Bgl*II and *Sma*I and microinjected into the male pronuclei of one-cell C57BL/6J wild-type (WT) mouse embryos using standard transgenic techniques to generate Tg mice. The CMV enhancer/promoter shows extensive cross-talk with other promoters because it contains many transcription factor binding sites [7,9–11,54]. However, as it is not a tissue-specific promoter, the protein expression of the *hPRDX4* transgene in each tissue will be affected by its site of integration into the mouse genome (i.e., by chance). C57BL/6J mice (Charles River Laboratories, Yokohama, Japan) were used as the control WT mice in our serial *in vivo* studies.

Our data show that, in RT-PCR, the expression of endogenous mouse PRDX4 (mPRDX4) is clearly recognized in every tissue of non-treated Tg and WT mice and is identified particularly strongly in the pancreas, testes, liver, and brain [7,9,10]. In contrast, the hPRDX4 expression in the Tg mice is exclusively enhanced, especially in the pancreas,

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aorta, liver, testes, heart, small intestine and brain [7,9–11]. In addition, a Western blotting analysis and immunohistochemical examination have demonstrated that hPRDX4 is particularly highly expressed in non-treated Tg tissues but is not expressed at all in non-treated WT mice [7–11]. Biochemical examinations show no remarkable difference between WT and Tg mice in whole-body glucose homeostasis, insulin resistance, or lipid profiles under basal conditions [7,10]. Furthermore, neither physiologically nor morphologically significant differences between the two groups of mice are evident.

Streptozotocin-induced Type 1 DM mouse model

To develop this model, WT and Tg male mice 8 weeks of age weighing 20 to 22 g are fasted for 18 h and intraperitoneally injected with a single high dose of streptozotocin (SHDS) (Sigma-Aldrich, Co., St. Louis, MO, USA) at 150 mg/kg body weight (BW), dissolved in sodium citrate buffer (pH 4.5). Mice with blood glucose levels higher than 250 mg/dL are considered to be DM. Sham mice are given the same volume of sodium citrate buffer.

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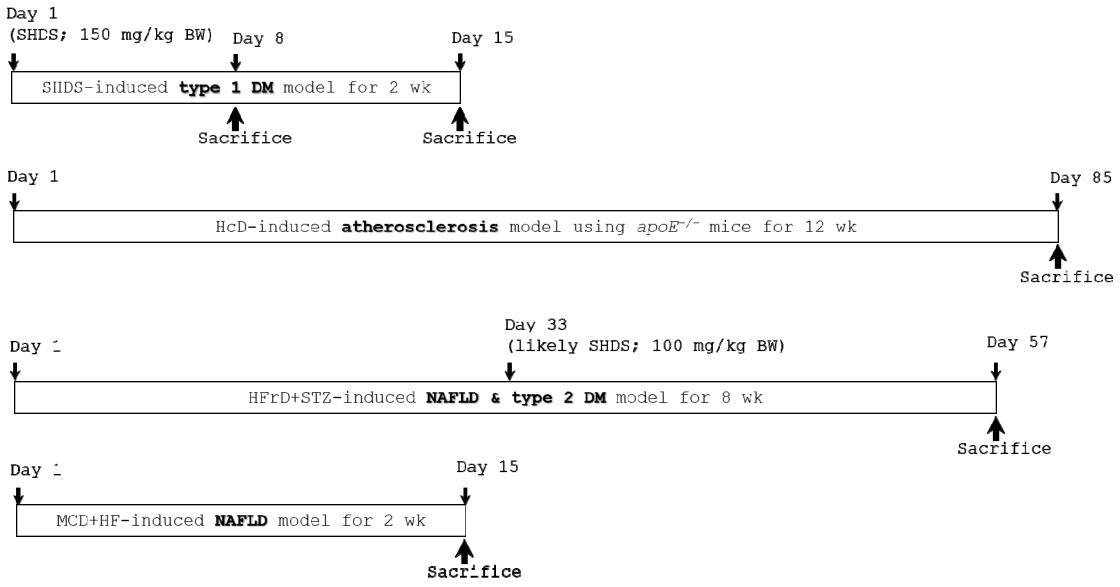
Animals are sacrificed 1 or 2 weeks after SHDS injection. The experimental procedure is summarized in Figure 2 as a schematic representation. The representative histopathology of the pancreas in type 1 DM mice is available in our previous studies [7,8], showing that the pancreatic islet areas are reduced along with CD3-positive T-lymphocyte infiltration and activated β-cell apoptosis.

Figure 2



Hypercholesterolemia-induced atherosclerosis mouse model

In contrast to lipid metabolism in humans, the lipoprotein profile in mice is high-density lipoprotein (HDL)-dominant; as such, WT mice are essentially resistant to high-cholesterol diet (HcD)-induced atheromatous plaque formation [9,26,33,34,37,39,55]. Thus, to achieve

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hypercholesterolemia-induced atheromatous formation, we generated Tg and *apolipoprotein E* (*apoE*)-knockout ($apoE^{-/-}$) mice ($hPRDX4^{+/+}/apoE^{-/-}$) by crossing *apoE*-KO mice [9]. These atherosclerotic model experiments were performed on 8-week-old male mice weighing 20 to 22 g. $ApoE^{-/-}$ and $hPRDX4^{+/+}/apoE^{-/-}$ mice are fed an HcD containing 1.25% cholesterol, 15.0% lard, and 0.5% sodium cholic acid (CA). They are sacrificed 12 weeks later [9,33,34,37]. The experimental procedure is summarized in Figure 2 as a schematic representation. The representative histopathology of mouse aortic atheroma is available in our previous studies [9,26,33,34,37], showing a variably thickened intima with the accumulation of foamy macrophages (Mϕs), migrating vascular smooth muscle cells (SMCs), T-lymphocyte infiltration, central necrotic lipid cores, collagen accumulation, and covering fibrous cap formation.

Special-diet-induced NAFLD and/or type 2 DM mouse models

Experiments were performed using 4- to 6-week-old male WT and Tg mice weighing

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approximately 18 g. These mice are fed a high-fructose diet (HFrD) (67% carbohydrates—98% of which is fructose—13% fat, and 20% protein; KBT Oriental Corporation, Saga, Japan) for 4 weeks to generate peripheral insulin resistance and NAFLD, followed by a single intraperitoneal injection (likely SHDS) of relatively low-dose (100 mg/kg BW) freshly prepared STZ (Sigma-Aldrich, Co.) dissolved in sodium citrate buffer (pH 4.5) after fasting for 18 h. The mice are then fed the same HFrD for a further four weeks, as described elsewhere [10,56]. The animals are sacrificed four weeks after STZ injection.

As additional NAFLD animal models, 8-week-old male WT and Tg mice are fed a methionine- and choline-deficient high-fat (MCD+HF) diet (60% fat; KBT Oriental Corporation) for 2 weeks, as described previously [9,10,57]. STZ is not injected in this model. The animals are sacrificed in a fed state two weeks later, and tissues—including the liver or small intestine—are excised.

In these NAFLD and/or type 2 DM (or insulin resistance) mouse models, food

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consumption is determined using metabolic cages obtained from SUGIYAMA-GEN CO., Ltd. (Tokyo, Japan). The experimental procedures are summarized in Figure 2 as a schematic representation. The representative histopathology of the liver, jejunum, or pancreas in NAFLD mice is available in our previous studies [9,10]. NAFLD in mice typically manifests as vesicular steatosis, chronic inflammation, hepatocellular ballooning, and fibrosis, associated with lipid deposition, increased expression of hepatic aminotransferase, stimulated hepatocytic apoptosis, and stellate cell activation, reminiscent of human NASH.

Novel, protective *in vivo* functions of PRDX4 in metabolic syndrome

PRDX4 protects against SHDS-induced type 1 DM by suppressing oxidative damage, inflammatory cytokines, and apoptotic activities throughout the course of the disease

We reported for the first time that [7,8], after SHDS-injection, Tg mice show significantly less hyperglycemia and hypoinsulinemia and a much faster response on the glucose

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tolerance test than treated WT mice, despite no marked differences in the insulin tolerance test. A histopathological observation showed that the Tg mice were significantly more resistant to serious SHDS-induced injury than WT mice and displayed accelerated reconstruction of the islets at two weeks while maintaining a high level of not only hPRDX4 but also endogenous mPRDXs expression, including mPRDX4. These data suggest that the overexpression of hPRDX4 in Tg mice protects against critical injury to the islets by SHDS while increasing the resistance (or reducing the vulnerability) of endogenous mPRDXs expression, reminiscent of a cascade of the PRDX family. In addition, hPRDX4 overexpression can suppress infiltration, particularly that of CD3-immunoreactive T lymphocytes.

STZ can be selectively accumulated in pancreatic β-cells via the low-affinity GLUT2 glucose transporter in the plasma membrane [58]. The transfer of the methyl group from STZ (i.e., SHDS) to the DNA molecule causes DNA damage, resulting in the fragmentation of the DNA and β-cell death (necrosis/apoptosis) [58]. Indeed, apoptosis is the main cause

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of selective β-cell death at the onset of type 1 DM, typically resulting from an autoimmune assault against β-cells by the infiltration of mononuclear T-lymphocytes and cytotoxicity- and inflammatory factor-induced processes [7,8,59–64]. We actually have shown that the expression of many inflammatory factors, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, TNF receptor (TNFR) 2, Toll-like receptor (TLR) 3/4, and NFκ-B, is significantly accelerated in WT mice after SHDS induction, while conversely, such inflammatory signaling is significantly reduced in the Tg pancreas. We can also verify the suppression of apoptotic cell morphology, terminal deoxynucleotidyl transferase end-labeling (TUNEL) staining and cleaved Caspase-3 activation in Tg islets [7]. Furthermore, the expression of 8-hydroxy-2′-deoxyguanosine (8-OHdG), as an oxidative stress marker [7,9,10,65–67], is much higher in WT and Tg islets after SHDS-injection than before treatment; however, at two weeks, the expression in the Tg mice is significantly lower than that in the WT mice.

Taken together, these findings strongly suggest that hPRDX4 plays a protective, key

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role against SHDS-induced injury by inhibiting endothelial dysfunction, proinflammatory cytokines and cytotoxic T-cell infiltration, as well as by preventing β-cell-derived ROS generation or scavenging of the generated ROS, particularly the extracellular pool of ROS. The insulin-secreting β-cells in the Tg islets, where hPRDX4 is specifically expressed, are significantly more protective against SHDS-induced insulitis and apoptosis than those of WT mice. Additionally, the Tg islets are more likely to activate the proliferation of β-cells, since the specific expression of hPRDX4 might be able to accelerate replication of pluripotent cells to repair and remodel islets [7,8].

Two different mechanisms have been proposed to explain this unique effect of PRDX4 in the repression of apoptosis. One mechanism involves the direct function of PRDX4 by scavenging the increased ROS/oxidative stress due to high glucose levels (DM). The other involves the indirect function of PRDX4 by preventing inflammatory cells (particularly, T lymphocytes) from infiltrating and/or reducing various proinflammatory mediators/cytokines and receptors/ligands involved in the cell survival and growth. PRDX4

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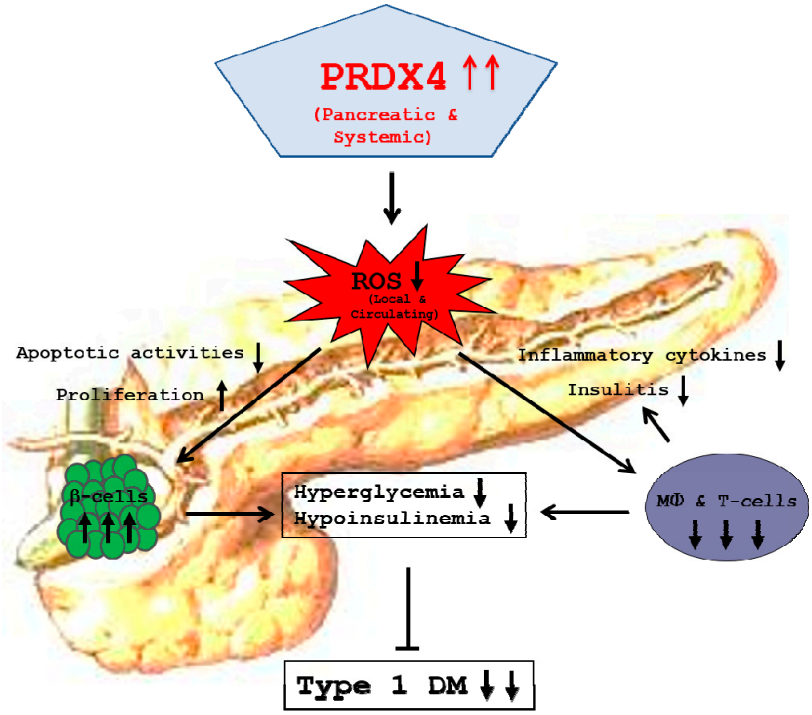
may be pathophysiologically relevant to the protection against type 1 DM in humans.

Figure 3 briefly summarizes the important roles of PRDX4 in this SHDS-induced type 1 DM mouse model.

Figure 3



PRDX4 protects against atherosclerotic progression in hyperlipidemia-induced atheromatous and vulnerable plaque formation by suppressing oxidative damage and apoptosis

In addition, our data [9] have confirmed the specific expressions of hPRDX4 *in vitro* and *in*

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vivo in aortic vascular SMCs and Mϕ isolated from *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice but not from *apoE*$^{-/-}$ mice. Intriguingly, we were able to show that the expression of the hPRDX4 transgene overcomes that of the endogenous mPRDX4 gene in the significantly suppressed atherosclerotic plaques of *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice. As an additive effect, the circulating serum levels of hPRDX4 protein are significantly elevated in *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice as well. Significantly decreased levels of circulating oxidant markers, serum thiobarbituric acid reactive substances (TBARS) [9–11,68], can also result in the subsequent suppression of endothelial dysfunction and repressed turbulence, supporting the protective effects of PRDX4 on attenuated atherosclerotic progression and strengthened stable plaque formation in *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice.

A detailed morphological observation shows that hypercholesterolemia-induced Mϕ-rich atherosclerosis is significantly reduced, although more SMC-rich plaques are evident in *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice than *apoE*$^{-/-}$ mice. Their atheromatous formation is characterized by a thicker fibrous cap, a greater amount of collagen, and fewer central

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necrotic lipid cores than *apoE*$^{-/-}$ mice, reminiscent of the structure of human stable plaques. As plaque vulnerability is known to be the most critical factor in cardiac attack and subsequently potential death [9,69], the specific induction of PRDX4 might be a potential therapeutic strategy to prevent atherosclerotic progression and to possibly improve plaque stability. Furthermore, the overexpression of hPRDX4 in the atherosclerotic aorta protects against chronic inflammation and the upregulation of pro-inflammatory cytokines by preventing inflammatory cell-derived ROS generation and scavenging the generated extracellular pool of ROS. Indeed, we detected lower local levels of 8-OHdG and oxidized LDL (ox-LDL) as well as systemic levels of TBARS in HcD-fed *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice than in *apoE*$^{-/-}$ mice. Finally, significantly fewer TUNEL-positive Mϕs, SMCs and endothelial cells (ECs) are noted in fewer atherosclerotic intimal lesions in *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice than *apoE*$^{-/-}$ mice, along with lower expression of Bax and Caspase-3, which are key apoptotic factors. Supporting those *in vivo* data, our *in vitro* experiments can show that the number of apoptotic cells including Mϕ is significantly

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lower in *hPRDX4*$^{+/+}$/*apoE*$^{-/-}$ mice than in *apoE*$^{-/-}$ mice after the stimulation with acetyl-LDL and Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor 58035 (i.e., free cholesterol-loading) [9].

Mϕ apoptosis may be an important feature of vulnerable plaque formation with an increased size of the necrotic lipid core due to weakened phagocytic clearance [34,69,70]. However, apoptosis of ECs is also critical, as the initial step in ROS-induced atherosclerosis involves endothelial damage, the increased expression of adhesion molecules, and inflammatory cell migration [2,6,34]. SMC apoptosis can lead to a loss of interstitial collagen fibers, resulting in unstable plaques that are prone to rupture [36,70,72–74]. Taken together, these findings suggest that, since oxidative stress is closely associated with apoptosis, PRDX4 may play a key role in ameliorating atherosclerotic progression in HcD-fed *apoE*$^{-/-}$ mice by protecting the aorta from oxidative stress and reducing apoptosis of various vascular cells.

In conclusion, accumulating data suggest that the overexpression of hPRDX4 plays a

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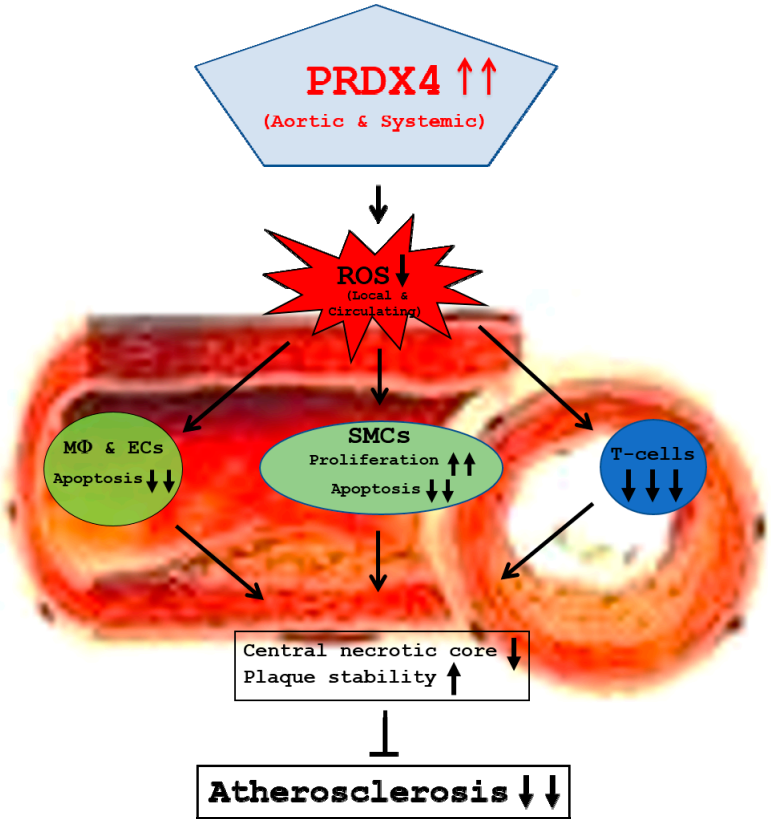
crucial role in (i) ameliorating atherosclerotic progression by reducing local and systemic oxidative stressors, decreasing the size of the central necrotic core by suppressing Mϕ apoptosis, and reducing inflammatory cell migration by suppressing EC apoptosis; and (ii) reducing possible markers of plaque instability by thickening the fibrous caps and increasing the collagen-rich matrix by suppressing SMC apoptosis. Figure 4 summarizes the pivotal roles of PRDX4 in the atherosclerotic mouse model.

Figure 4



PRDX4 protects against the development of NAFLD, type 2 DM, and metabolic syndrome

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by ameliorating oxidative stress-induced injury in a nongenetic mouse model by feeding mice an HFrD after injecting STZ

Our laboratory detected the localized expression of hPRDX4 exclusively in the liver of Tg mice by PCR, Western blotting, and immunohistochemistry after establishing an HFrD+STZ-induced nongenetic mouse model [10]. The CMV enhancer/promoter in the hPRDX4 transgene is readily stimulated by ROS and chronic inflammation during the progression of mouse NAFLD and insulin resistance via cross-talk with other promoters for other transcription factor binding sites, such as the two activator protein 1s (AP1s), four NFκBs, and five cAMP response element binding proteins (CREBs) [9–11,54]. The intrahepatic overexpression of hPRDX4 is therefore feasible in the model Tg mice. We also found that, similar to the above atherosclerosis model, the expression of this activated transgene can overcome that of the endogenous PRDX4 gene in modest NAFLD livers of Tg mice. In addition, the circulating (i.e. systemic) serum levels of soluble hPRDX4 are significantly elevated in model Tg mice. In that sense, the model establishment accelerates

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oxidative stressors by increasing hepatic and blood ROS levels in HFrD+STZ-induced type 2 DM, NAFLD, and possibly metabolic syndrome, as shown by the intrahepatic expression of 8-OHdG, 4-hydroxy-2-nonenal (4-HNE), and systemic serum levels of TBARS, respectively.

The WT but not Tg mice showed particularly dramatic increases in blood glucose and insulin levels, as well as delayed glucose clearance, after STZ injection. This overt dysfunction of the glucose/insulin metabolism may be due to the significant reduction in the insulin sensitivity, especially in the liver, due to significantly accumulated triglyceride (TG) and free fatty acid (FFA) levels, manifesting as the establishment of NAFLD. The extreme antioxidative state and suppressed NAFLD in the model Tg mice are closely associated with fewer intrahepatic T lymphocytes and Kupffer cells and lower alanine aminotransferase (ALT) and expression of TNF-α and its receptor (TNFR). Taken together, these findings indicate that PRDX4 suppresses the progression of simple steatosis to NASH by blocking not only the ‘first hit (disordered hepatic lipid accumulation)’ but also the ‘second hits

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(metabolic, cytokine, and oxidative stressors)', according to the 'two-hit' hypothesis [75,76], and should subsequently protect against the initiation to progression of type 2 DM.

Regarding the third hit—apoptosis—our *in vivo* evidence has shown significantly fewer apoptotic hepatocytes in model Tg livers than model WT livers, similarly accompanied by reduced expression of the pro-apoptotic markers Bax and cleaved Caspase-3. Our supportive *in vitro* data further show that oxidative stress (H_2O_2)-induced apoptosis is significantly reduced in cultured hepatocytes obtained from Tg mice. In addition to the anti-apoptotic effects of PRDX4, the model Tg liver also demonstrates significantly reduced expression of α-smooth muscle actin (α-SMA), a marker of stellate cell activation, and of collagen type I, a marker of hepatic fibrosis/fibrogenesis. Indeed, as physiologically aberrant apoptosis of hepatocytes is known to trigger the uncontrolled activation and differentiation of stellate cells into myofibroblasts and subsequent liver fibrogenesis/fibrosis, apoptotic hepatocytes potentially provide a critical 'third hit' that might drive the progression from NASH to cirrhosis and HCC [76].

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In conclusion, PRDX4 overexpression plays critical key roles in (i) ameliorating the local (intrahepatic) and systemic (circulating) expression of oxidative stressors, (ii) preventing the initiation of NAFLD and insulin resistance by reducing hepatic TG and FFA accumulation, and (iii) protecting against the development of obesity, NASH, and type 2 DM by suppressing chronic inflammation, apoptosis, and fibrogenesis, with its unique intracellular and extracellular effects, as summarized in Figure 5. Our observations support the utility of activators of PRDX4 as therapeutic agents for ameliorating visceral obesity, NASH, and/or type 2 DM (i.e. metabolic syndrome) by suppressing oxidative damage and inflammatory signaling and by improving liver insulin sensitivity throughout the course of these diseases.

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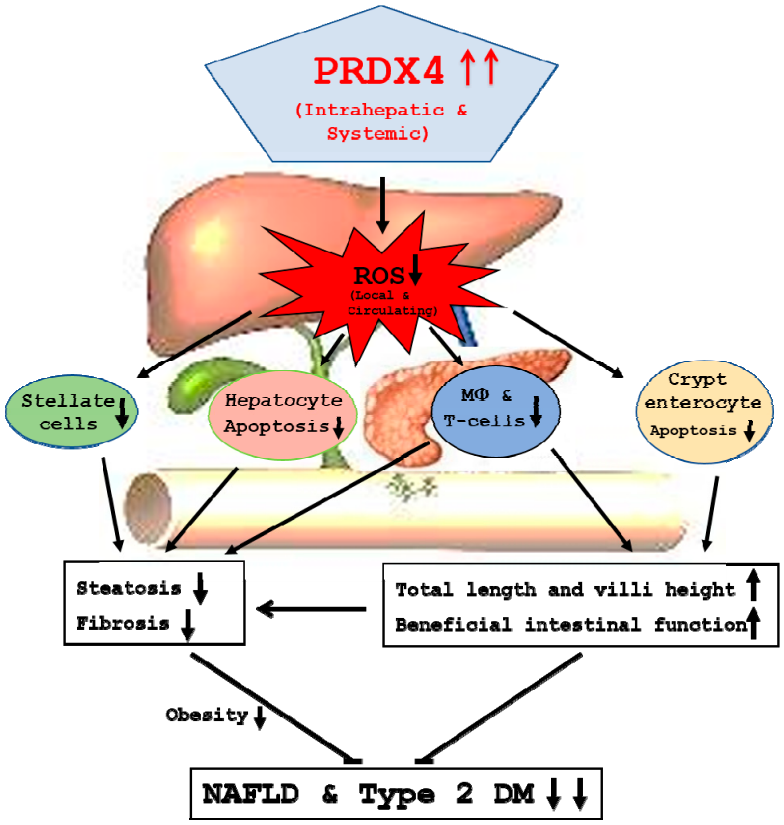
Figure 5



PRDX4 exerts beneficial effects on not only the hepatic but also the intestinal function by protecting against oxidative stress-induced injury in an MCD+HF-induced NAFLD mouse model

In our second MCD+HF diet-induced NAFLD mouse model [11], an accumulating body of our data have confirmed that the overexpression of intracellular (local) and secreted (systemic) PRDX4 significantly reduces or even wholly prevents damage due to NAFLD. We also provide the first evidence that the small intestine as well as the liver are spared by

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the antioxidant properties of PRDX4 and that the overexpression of PRDX4 beneficially affects the intestinal function while preventing the progression of NAFLD. In addition to the target organ (the liver), the small intestine is also involved in a key role in the etiology of NASH, which is one aspect of human metabolic syndrome, manifesting as visceral obesity, dyslipidemia, type 2 DM, and atherosclerosis [8–11,14]. Since the intestine is the first organ to encounter nutrients and serves as gatekeeper at the pathophysiological interface between the body and the diet, it can play a varied but crucial role in the metabolic processing of nutrients along with efficient lipid and bile acid (BA) absorption, partly via enterohepatic circulation [22,23].

Indeed, detailed morphological, biochemical, and molecular observations have shown that, very similarly to findings in other mice models of metabolic syndrome, the specific expression of hPRDX4 in the small intestine as well as the liver of Tg mice is evident exclusively after establishing this MCD+HF dietary mouse NAFLD model. We have further shown that model Tg mice display a phenotype of significant resistance to local and

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systemic inflammation/oxidative injury along with markedly higher levels of tissue (i.e. liver and intestine) PRDX4 and circulating serum soluble PRDX4 protein. Indeed, the Tg mice have shown a reduction in oxidation by decreasing the hepatic, intestinal, and circulatory ROS levels, even after the establishment of MCD+HF feeding-induced NAFLD and intestinal dysfunction, as demonstrated by a reduced level of DHE binding in hepatocytes/enterocytes and of TBARS in serum.

Our laboratory noted the unique finding that intestinal lipid deposits in surface enterocytes, derived from TG, FFA, and cholesteryl ester (CE) accumulation, are significantly increased in model Tg mice, accompanied by the presence of fewer apoptotic and more proliferating epithelial cells, particularly in the crypts, than model WT mice. It has been suggested that, inversely, activated apoptosis of the intestinal multipotent stem cells can induce uncontrolled cell-renewal processes, including migration or differentiation, and the subsequent shortening of the jejunal villi height and the total length [11,77]; however, this has not actually been confirmed in model Tg mice. This dysregulated renewal

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process may also significantly reduce the intestinal fat absorption capacity at the mucosal surface, where nutrient absorption mainly takes place [39]. Taken together, these findings support the above characteristic histopathological features of intestinal lipid accumulation in Tg mice. Furthermore, we observed fewer infiltrating jejunal macrophages, associated with a lower expression of various inflammatory cytokines, in the intestines of model Tg mice than in those of model WT mice. The stimulation of inflammation and apoptosis in the intestinal epithelium plays an important role in disrupting the mucosal integrity and barrier function protecting against enterobacterial invasion [78–80]. A study of human NASH patients cited a higher prevalence of small intestine bacterial overgrowth (SIBO) and a lower proportion of the phyla *Bacteroidetes* than healthy adult volunteers, with increased gut permeability [79]. We are going to perform further experiments to address these complicated issues, including assessing the relationship between the progression of metabolic syndrome and the intestinal bacterial counts and composition of the phyla/genera.

In conclusion, our serial *in vivo* study of the initiation and progression of NAFLD

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provides new evidence supporting several potential mechanisms by which PRDX4 overexpression plays critical, key roles in (i) reducing local (intrahepatic and intraintestinal) and systemic (circulating) oxidative stressors; (ii) affecting beneficial intestinal function by suppressing crypt enterocyte apoptosis and inflammation and by protecting against the shortening of the total length and villi height; and (iii) synergistically reducing the severity of NAFLD. Both the small intestine and liver can be spared to some degree by the characteristic antioxidant properties of PRDX4. Specific activators of PRDX4 may therefore offer therapeutic potential for ameliorating the progression of NAFLD and intestinal dysfunction by attenuating oxidative damage. Figure 5 briefly summarizes the crucial *in vivo* roles of PRDX4 in these mouse NAFLD models.

Intriguing topics for future studies and concluding remarks on our unique PRDX4 Tg mice

Metabolic syndrome is a complex, multifactorial disease, orchestrated by diet type affecting

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glucose/lipid/BA metabolism, oxidative stress, insulin resistance, inflammatory cell infiltration, cytokine levels and/or apoptotic activities in various organs, translocation of either intestinal bacteria or microbial cell components, and other factors. There is a growing body of evidence supporting the innate link between metabolic syndrome and the only known secretory member of the PRDX antioxidant family (PRDX4), which has not only local but also whole-body functions. Therefore, clinically, specific accelerators of PRDX4 may prove useful as therapeutic agents for protecting against the initiation and progression of DM, atherosclerosis, NAFLD, and subsequently metabolic syndrome in humans by suppressing oxidative damage and pro-apoptotic or inflammatory factors throughout the course of these diseases. Our unique Tg mouse model will be useful for studying the associations of the locally and systemically antioxidant properties of PRDX4 overexpression with lipid/BA/glucose metabolism and may also be a promising novel animal model of human metabolic syndrome.

We plan to develop another *in vivo* model of liver dysfunction regarding bile duct

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ligation (BDL) using both Tg and WT mice in order to evaluate the roles of PRDX4, with a focus on BA metabolism. As described elsewhere, to produce a ligation-induced cholestatic liver injury (BDL) model for two weeks, the peritoneal cavity is opened after a midline upper-abdominal incision, and the common bile duct is double-ligated with sterile surgical silk sutures and cut between the ligatures in 2 groups of mice at 6–8 weeks of age under anesthesia [40,81]. Since BA/lipid metabolism strongly correlates with the pathogenesis of metabolic syndrome, including atherosclerosis and/or NAFLD, the establishment of BDL mice model is critical for clarifying its biochemical/molecular mechanisms in human cholestatic liver injury diseases as well as potentially atherosclerosis and NAFLD. Indeed, BAs are known to be synthesized in hepatocytes from lipids and cholesterol and to contain variable types of cholic acid (CA), and each BA demonstrates a specific affinity to BA receptors and functions as a signaling molecule with distinctive effects inducing oxidative stress, cytotoxicity, and/or apoptosis [38,39,82]. It has been suggested that metabolic syndrome may have a close correlation with higher levels of secondary BAs, such as

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deoxy-CA, resulting from major changes in the intestinal microbial flora, at least in part [39,83]. Our laboratory has therefore been focusing on the early-phase BA metabolism and antioxidative response mediated intra- and extra-cellularly by PRDX4 in cholestatic Tg mice, developing a detailed BA profile and examining the function of the liver and small intestine.

However, it would also be very intriguing to further study the relationships between PRDX4 and the inhibition of cancer progression for HCC. NAFLD—an umbrella term covering a broad spectrum of clinicopathological features, ranging from simple steatosis to severe NASH—is closely involved in the development of metabolic syndrome, and NASH occasionally progresses to end-stage liver diseases, including cirrhosis and/or HCC [14,18]. Diethylnitrosamine (DEN), a genotoxic drug with well-established uses in animal models to induce hepatocarcinogenesis, is used as a carcinogen in a murine HCC model [84,85]. A long-term, weekly administration (35 mg/kg BW) is used to establish HCC in male WT C57BL/6J mice after 20–35 weeks. In agreement with our hypothesis, oxidative stressors

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can also cause carcinogenesis through the induction of DNA damage and lipid dysregulation [86–88]. Indeed, the PRDX4 expression is variably high in most human malignant neoplasms, including prostate/lung adenocarcinoma or glioblastoma, and surprisingly, the suppression of PRDX4 leads to a significant reduction in tumor growth and/or metastatic potential [88–93]. In line with those findings, our data from clinical samples [10] show that patients with type 2 DM have significantly higher serum hPRDX4 levels than healthy adult volunteers, likely due to the greater demand for antioxidant defense in this chronic inflammatory disease. The unknown mechanisms underlying the antioxidative function of PRDX4 and cancer progression in HCC are an intriguing topic for researchers to explore in the future.

A recently published paper from another Japanese laboratory using *PRDX4* knockout mice (*PRDX4*$^{-/y}$), whose spermatogenic cells are more susceptible to apoptosis via oxidative damage than those of WT *PRDX4*$^{+/y}$ male mice, reported that the levels of PRDX4 protein in *PRDX4*$^{+/y}$ male mice were significantly higher in nearly all tissues, especially those of the

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testes and pancreas, than those in *PRDX4*$^{-/y}$ male mice [94]. Considering the particularly mild oxidative stress phenotype, and no obvious phenotype, except for the defects in spermatogenesis, of *PRDX4*$^{-/y}$ male mice, and its low antioxidative capacity [94], it is possible that the observed *in vivo* (beneficial) effects in our unique Tg mice might rely on other function (including redox signaling) rather than neutralizing H_2O_2. In this context, we can suggest that human PRDX4 (hPRDX4) should play a more crucial role in antioxidants and redox signaling than mouse PRDX4 (mPRDX4), even though the amino acid sequence of mPRDX4 shows very high homology to that of hPRDX4 (>89%), as determined by the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD, USA). Nevertheless, in that vein, our ongoing studies use the animal metabolic syndrome models described above with *PRDX4*$^{-/y}$ male mice to further confirm the diverse, critical *in vivo* roles of PRDX4 in protecting against the initiation and progression of chronic inflammatory diseases.

We recently established a novel model for studies of HcD-induced atherosclerosis and

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NAFLD using the world's smallest Microminipigs™ (μMPs; Fuji Micra Inc., Shizuoka, Japan) [95–97]. Unlike rodents or rabbits, swine represent a promising, useful experimental animal model, since their lipoprotein metabolism as well as their anatomy, physiology, and feeding and sleeping habits are quite similar to those of humans. Thus, one of our future aims is to clarify the pathogenic and molecular mechanisms underlying the antioxidative properties of PRDX4 during the initiation and development of metabolic syndrome, particularly in μMPs.

Abbreviations

Apo, apolipoprotein; ALT, alanine aminotransferase; BA, bile acid; BW, body weight; CA, cholic acid; CE, cholesteryl ester; CMV, cytomegalovirus; Cys, cysteine; DHE, dihydroethidium; DM, diabetes mellitus; EC, endothelial cell; ER, endoplasmic reticulum; FFA, free fatty acid; HCC, hepatocellular carcinoma; HcD, high-cholesterol diet; H&E, hematoxylin and eosin; HF, high fat diet; HFrD, high fructose diet; H_2O_2, hydrogen

peroxide; hPRDX4, human peroxiredoxin 4; IL, interleukin; LDL, low-density lipoprotein; Mϕ, macrophage; MCD+HF, methionine- and choline-deficient high fat diet; MDA, malondialdehyde; mPRDX4, mouse peroxiredoxin 4; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NFκ-B, nuclear factor κ-B; O_2^-, superoxide anion; PDI, protein disulfide isomerase; PRDX, peroxiredoxin; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SHDS, single high dose of streptozotocin (STZ); SMC, smooth muscle cell; STZ, streptozotocin; Tg, hPRDX4 transgenic; TG, triglyceride; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase end-labeling; WT, wild-type; μMPs, microminipigs; 4-HNE, 4-hydroxy-2-nonenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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Disclosure Statement

The authors declare no conflicts of interest in association with this study.

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Figure legends

Fig. 1. The pathway of the detoxification of reactive oxygen species (ROS) by peroxiredoxin (PRDX). O_2 is a highly reactive molecule and can easily form O_2^- (superoxide), subsequently undergoing dismutation to form H_2O_2 (hydrogen peroxide). The functional peroxidase activity of PRDXs is dependent on reduced forms of thioredoxin (redTRX) and/or glutathione (GSH). PRDX: peroxiredoxin, SOD: superoxide dismutase, GSSG: oxidized GSH, GPX: GSH peroxidase, ·OH: hydroxyl radicals, CoQ: coenzyme Q, p450: cytochrome p450, FAD: flavin adenine dinucleotide.

Fig. 2. A schematic presentation of the experimental procedures in various mouse models of metabolic syndrome using our unique Tg mice.

SHDS: single high dose of streptozotocin (STZ), HcD: high-cholesterol diet, apoE: apolipoprotein E, HFrD: high fructose diet, MCD+HF: methionine- and choline-deficient high fat diet.

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Fig. 3. Summary of the critical roles of PRDX4 in type 1 DM. This diagram depicts the crucial, protective roles of PRDX4 in the SHDS-induced type 1 DM mouse model.

PRDX4: peroxiredoxin 4, ROS: reactive oxygen species, DM: diabetes mellitus, SHDS: single high dose of streptozotocin (STZ), Mϕ: macrophage.

Fig. 4. Summary of the critical roles of PRDX4 in atherosclerosis. This diagram depicts the beneficial, protective roles of PRDX4 in the HcD-induced atherosclerosis mouse model.

PRDX4: peroxiredoxin 4, ROS: reactive oxygen species, Mϕ: macrophage, EC: endothelial cell, SMC: smooth muscle cell.

Fig. 5. Summary of the critical, protective roles of PRDX4 in NAFLD and type 2 DM. This diagram depicts the critical, protective roles of PRDX4 in the nongenetic

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(HFrD+STZ-induced) mouse model of NAFLD and type 2 DM, and the MCD+HF-induced NAFLD mouse model.

PRDX4: peroxiredoxin 4, ROS: reactive oxygen species, Mϕ: macrophage, NAFLD: nonalcoholic fatty liver disease, DM: diabetes mellitus.

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hPRDX4: human peroxiredoxin 4 (PRDX4), SHDS: single high dose of streptozotocin (STZ), DM: diabetes mellitus, HcD: high-cholesterol diet, HFrD+STZ: high fructose diet plus streptozotocin, NAFLD: nonalcoholic fatty liver disease, MCD+HF: methionine- and choline-deficient high fat diet, ROS: reactive oxygen species, Mϕ: macrophage, SMC: smooth muscle cell, EC: endothelial cell.