[Review]

Peroxiredoxin 4: Critical Roles in Inflammatory Diseases

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Abstract : We review the critical roles of peroxiredoxin (PRDX) 4 in inflammatory diseases. The PRDX family, a new family of proteins with an antioxidative function, is ubiquitously synthesized and abundantly identified in various organisms. The function of these enzymes, which include at least six distinct PRDX genes expressed in mammals, still remains unclear. Especially, in contrast to the intracellular localization of other family members, PRDX4 is the only known secretory form located in the extracellular space and exerts its protective function against oxidative damage by scavenging reactive oxygen species in the vascular vessels. To date, however, it is not clear whether or how PRDX4 expression affects various diseases *in vivo*. More recently, we generated human PRDX4 (hPRDX4) transgenic (Tg) mice, and, for the first time, established a type 1 diabetes mellitus model induced by a single high dose of streptozotocin on Tg mice. Our published data demonstrate that streptozotocin-treated Tg mice, which overexpress hPRDX4 in pancreatic islets, can protect pancreatic β -cells against streptozotocin-induced injury (insulitis) by suppressing increased oxidative stress and inflammatory signaling activation. These observations indicate that Tg mice could become a useful animal model to study the relevance of oxidative stress to inflammation, and that a specific accelerator of PRDX4 might prove to be a potential therapeutic agent for ameliorating various chronic inflammatory diseases.

Key words : peroxiredoxin 4, PRDX 4, inflammatory disease, human PRDX4 transgenic mice.

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Introduction

A growing body of evidence has shown that oxidative stress including oxidized molecules and reactive oxygen species (ROS), such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), and the concomitant generation of nitric oxide (NO), contributes to the initiation and progression of chronic inflammatory diseases [1–5]. Actually, oxygen (O_2) is a highly reactive molecule and can easily form the one-electron reduction state of O_2^- , after which it undergoes dismutation to form H_2O_2 , as shown in Fig. 1. For instance, increased oxidative stress within the extracellular space, i.e., vasculature, is a risk factor of atherosclerosis, considered to be a chronic inflammatory process, which is one of the main research themes in our laboratory. Oxidative stress also commonly plays a crucial role in atherosclerosis manifested by the accumulation of oxidized low-density lipoprotein (oxLDL) as well as the formation of ROS [4–7].

Peroxiredoxin (PRDX), a new family of proteins with an antioxidative function, is ubiquitously synthesized and abundantly identified in various organisms [1, 8]. These enzymes, which include at least six distinct PRDX genes expressed in mammals, contain a reactive Cys in a conserved region near the N-terminus that forms cysteine-sulfenic acid as an intermediate reaction during the reduction of H_2O_2 , and functional peroxidase activity is dependent on reduced forms of thioredoxin (redTRX) and/or glutathione (GSH) [8, 9]. Figure 1 summarizes the pathway of

Fig. 1. The pathway for detoxification of reactive oxygen species (ROS) by peroxiredoxin (PRDX). O_2 is a highly reactive molecule and can easily form O_2^- and hydroxyl radicals ($\cdot OH$), and then undergoes dismutation to form H_2O_2 . Functional peroxidase activity of PRDXs is dependent on reduced forms of thioredoxin (redTRX) and/or glutathione (GSH). SOD: superoxide dismutase, oxTRX: oxidized TRX, TR: TRX reductase, NADPH: nicotinamide adenine dinucleotide phosphate, GSSG: oxidized GSH, GPX: GSH peroxidase, GR: GSH reductase.

the detoxification of ROS by PRDXs. The PRDXs still remain functionally unclear. Several studies have shown, however, that PRDXs suppress downstream signaling responses of receptors, such as apoptosis [9, 10]. *In vivo*, PRDX1 attenuates excessive endothelial activation and atherosclerosis [11]; PRDX1 deficiency worsens the susceptibility to multiple cancers with aging [12]; loss of PRDX2 is sufficient to influence the replication and migration of smooth muscle cells (SMCs) during vascular remodeling [13]; and PRDX6 knockout mice (PRDX6^{-/-}) with C57BL/6 and 129/SvJ mixed backgrounds fed a high fat diet show more significantly pronounced atherosclerotic lesions than controls [14]. Thus, these data allow us to hypothesize that PRDX can function not only in an antioxidative signaling but also in other protective cascades against various types of pathological processes, including atherosclerosis.

Among the PRDX family, human PRDX4 (hPRDX4) is the only known secretory form located in not only the intracellular 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 the mitochondria) [8, 15, 16]. However, despite the regulatory role of PRDX4 in the nuclear factor κ B (NF- κ B) [17], epidermal growth factor and p53 [18] or thromboxane A2 receptor [15] cascade shown in several previous studies, the pathological and physiological relevance of PRDX4 remains unclear. PRDX4 is known to exert its protective function against oxidative damage by scavenging reactive oxygen species (ROS) in the extracellular space [1, 16, 17], such as in the vascular vessels ranging from small to large sizes, where atherosclerotic lesions are called “arteriosclerosis” or “atheroma”. Additionally, as to the former “arteriosclerosis”, diabetes is closely related to its pathophysiological features. Therefore, we hypothesized that this unique PRDX4 *in vivo* plays a pivotal role in the protection against the destruction of pancreatic β -cells during the development of diabetes. More recently, we generated hPRDX4 transgenic (Tg) mice and evaluated the role of PRDX4 in a type 1 diabetes mellitus (T1DM) model induced by a single high dose of streptozotocin (SHDS), displaying a specific disruption of β -cells accompanied by increased proinflammatory cytokines and ROS production [1]. Indeed, it is known that the majority of T1DM cases can be attributed to autoimmune-mediated specific β -cell apoptosis, which is generally accepted to be an inflammatory disease of the pancreatic islet cells, also known as ‘insulinitis’ [19, 20].

In this review, we focus on the crucial roles of PRDX4 in various inflammatory diseases, especially in DM and atherosclerosis, and demonstrate that our Tg mice should become a useful animal model to study the relevance of oxidative stress to inflammation, and, additionally, ROS- and/or inflammation-induced chronic diseases.

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

Tg mice were generated as described before [1]. hPRDX4 cDNA was amplified by RT-PCR and cloned into the pGEM-T easy vector system (Invitrogen, Life Technologies Japan Ltd., Tokyo, Japan). Its primers were based on a published sequence (Genebank accession no. NM_006406). The entire nucleic acid sequence, containing a 0.6 Kb cytomegalovirus (CMV) enhancer/promoter, 0.8 Kb hPRDX4 cDNA, and 0.2 Kb bovine growth hormone polyadenylation

(BGHPA), was purified by restriction enzyme digestion with *Bgl*II and *Sma*I, and was microinjected into the male pronuclei of one-cell C57BL/6 mouse (Charles River laboratories, Yokohama, Japan) (as the control wild-type (WT) mice) embryos by standard transgenic technology [1].

The data showed that, in PCR, the expression of endogenous mouse PRDX4 (mPRDX4) was clearly recognized in every tissue of the non-treated Tg and WT mice, and was observed particularly in the pancreas, testis, liver and brain. The hPRDX4 expression in the Tg mice was highly enhanced especially in the pancreas, aorta, testis, heart and brain. Moreover, Western blotting analysis revealed that hPRDX4 was markedly expressed in non-treated Tg pancreas, and to a lesser amount in the testis. Immunohistochemical examination demonstrated that hPRDX4 was particularly expressed in non-treated Tg islets including β - and α -cells, ductal epithelium, and a small number of acinar cells, whereas it was not expressed in non-treated WT mice. Additionally, a small number of spermatogenic cells from the testis of Tg mice only were specifically positive for hPRDX4, and smooth muscle cells (SMCs) in the Tg aorta and columnar epithelial cells in the Tg large intestine also revealed a weak expression of hPRDX4. No WT tissues displayed any expression of hPRDX4. On the other hand, the pancreatic islet area from the non-treated WT and Tg mice showed no significant difference, and the percentage of insulin-positive cells in the non-treated WT islet was very similar to that of the Tg mice (more than 85%). Biochemical examinations also revealed no difference between WT and Tg mice in whole-body glucose homeostasis or insulin resistance under basal conditions. No morphological or biochemical difference between the two groups of mice was seen in the testes, either.

In contrast, in a recently published paper from another laboratory using PRDX4 knockout mice (PRDX4^{-/-}), whose spermatogenic cells were more susceptible to apoptosis via oxidative damage than those of wild-type PRDX4^{+/+} male mice, PRDX4 protein in PRDX4^{+/+} male mice was present at much higher levels in nearly all tissues, especially in the testis and pancreas [21]. These findings were analogous to our above results obtained by Western blotting and immunohistochemical analyses.

Single High Dose of Streptozotocin (SHDS)-induced Type 1 Diabetes Mellitus (T1DM) Mouse Model

After SHDS-injection (intraperitoneal injection at 150 mg/kg body weight [1]), the WT mice showed a dramatic increase in blood glucose, a decrease in blood insulin levels and a delay of glucose clearance. This damage to glucose/insulin metabolism can be attributed to a disruption of pancreatic islets by SHDS (Fig. 2A). The Tg mice, however, had significantly less hyperglycemia and hypoinsulinemia, along with a much faster response on the glucose tolerance test compared with the treated WT mice, even though there was no significant difference in insulin resistance. Morphological observation showed that SHDS caused serious injury to the islet areas that were dramatically diminished in animals 1 or 2 wk after the injection (Fig. 2A, B). However, compared with the WT mice, the Tg mice revealed a resistance to injury and an accelerated reconstruction of the islets 2 wk after SHDS-injection (Fig. 2A, B), along with maintaining a high level of not only hPRDX4 (Fig. 2C, D) but also endogenous mPRDXs expression, includ-

Fig. 2. Alterations of islet areas and hPRDX4 expression in WT and Tg pancreas after SHDS-injection. A) and B) Pancreatic islet area showed no significant difference in the WT and Tg mice at 0 and 1 wk after SHDS-injection (0 wk; WT $24.0 \pm 1.9 \times 10^3 \mu\text{m}^2$ vs. Tg $24.9 \pm 2.5 \times 10^3 \mu\text{m}^2$ and 1 wk; WT $5.8 \pm 0.5 \times 10^3 \mu\text{m}^2$ vs. Tg $6.3 \pm 0.5 \times 10^3 \mu\text{m}^2$). WT islet area continuously decreased during the first 2 wk, whereas the Tg islet area significantly recovered at 2 wk ($9.2 \pm 0.8 \times 10^3 \mu\text{m}^2$) compared with that of 1 wk after SHDS-injection. Therefore, the Tg islet area was significantly larger by 2.2-fold than that of the WT ($4.2 \pm 0.4 \times 10^3 \mu\text{m}^2$) 2 wk after SHDS-treatment. C) In immunohistochemistry, there was little or no expression of hPRDX4 in the WT islets, whereas the Tg islets expressed a much higher level of that after SHDS-injection 1 and 2 wk. D) In real-time PCR, hPRDX4 was observed specifically in the Tg pancreas during the 2 wk, and its expression decreased 1 or 2 wk after SHDS-injection. *: $P < 0.05$, ***: $P < 0.0001$.

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ing mPRDX4. This morphologic observation was in parallel with the above biological results. Therefore, these data indicated that overexpression of hPRDX4 in the Tg mice protected against critical injury to islets by SHDS together with more resistant or less vulnerable endogenous mPRDXs expression, reminiscent of a cascade of the PRDX family. This expression pattern restored the damage of glucose/insulin metabolism and amelioration of injured islets, as observed in the WT mice.

Furthermore, the number of CD3-positive T-cells increased in the islets injured by SHDS, indicating that the inflammatory response in this insulinitis is attributable to increased infiltrating CD3-positive T-cells. Thus, the suppression of CD3-positive T-cell infiltration in Tg mice has a special implication in this model. These results show that hPRDX4 can suppress an infiltration particularly of CD3-positive T lymphocytes. On the other hand, apoptosis is the main cause of selective β -cell death at the onset of T1DM, which is typically caused by an autoimmune assault against β -cells with infiltration of mononuclear cells [22, 23]. With regard to apoptosis, the mechanisms of apoptosis involve direct T-cell cytotoxicity- and inflammatory factor-induced processes in insulinitis [20, 22–26]. These inflammatory cytokines can upregulate Fas expression in β -cells, thereby facilitating cell recognition via Fas/Fas ligand, activate the β -cell gene networks under the control of the transcription factors, including NF κ -B and signal transducer and activator of transcription-1 (STAT1), and induce ROS, inducible NO synthase (iNOS) and NO production, exacerbating apoptosis via Caspase activation [22–25, 27, 28]. Actually, we demonstrated that the expression of many inflammatory factors was significantly activated in the WT mice after SHDS-induction, and conversely, such inflammatory signaling was significantly down-regulated in the Tg pancreas. As also shown in our data, particularly and classically, interleukin (IL)-1 β has a more pronounced pro-apoptotic effect in the β -cells, and some reports have demonstrated that *in vitro* exposure of β -cells to IL-1 β or to IL-1 β + interferon (IFN)- γ causes elevated proinsulin/insulin levels and a preferential loss of first-phase insulin secretion in response to glucose, which is very likely in diabetic patients [29]. Besides, the expression of 8-hydroxy-2'-deoxyguanosine (8-OHdG), as an oxidative stress marker [30–32], was much higher in the WT and Tg islets after SHDS-injection than before treatment in immunohistochemistry (Fig. 3A), supporting the premise that activated ROS production is not only induced by up-regulated cytokines from stimulated T-cells, but also is derived from β -cells themselves in increased glucose concentration [3] in this diabetes model. At 2 wk, expression in the Tg was significantly suppressed, as compared with that in the WT mice (Fig. 3A). These results strongly suggest that hPRDX4 plays a protective role against injury by inhibiting 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. Compared with those of the WT, the down-regulated findings of terminal deoxynucleotidyl transferase end-labeling (TUNEL) staining and Caspase-3 activation in Tg islets verified (Fig. 3B) and supported these suggestions.

Interestingly, we demonstrated new pathological findings that the proportion of glucagon-positive cells in both the Tg and WT was markedly increased by about 64% after SHDS-induced islet destruction. Consequently, proliferating glucagon-positive α -cells, which were merely minor elements in less than 15% under basal conditions, became a predominant component of the islets.

Fig. 3. Immunohistochemical expression of oxidative stress marker, 8-OHdG, and apoptotic factors in WT and Tg pancreas. A) The expression of 8-OHdG was very weak in the untreated mice islets. However, at 2 wk after SHDS-injection, the expression was significantly increased in the pancreas of both groups. Additionally, 8-OHdG expression was significantly suppressed in the Tg mice as compared with that in the WT mice. B) In TUNEL staining, the percentage of TUNEL-positive apoptotic β -cells per islet was significantly lower in the Tg ($1.0 \pm 0.6\%$) than that of the WT mice ($3.2 \pm 0.7\%$). Additionally, the percentage of Caspase-3-positive cells was significantly decreased in the Tg ($2.7 \pm 1.2\%$) compared with that of the WT mice ($9.7 \pm 3.2\%$). *: $P < 0.05$.

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That data indicated that the replication of α -cells compensated for the significantly decreasing numbers of β -cells due to insulinitis and apoptosis. In addition, surprisingly, replicating β -cells in the Tg islets partially overlapped with replicating α -cells by double immunostaining techniques, which was confirmed by the data that adding the proportion of α - and β -cell from the Tg tissue exceeded more than 100%. In contrast, a proportion of less than 100% was noted in the WT islets. Based on these results, we proposed that the regenerative and neogenetic islet cells may be immature and pluripotent, and as a result, both glucagon and insulin would be immunoreactive with one part of many replicating cells, especially in Tg mice. Indeed, recent studies have revealed that a rapid course of islet hyperplasia after streptozotocin-induced injury depends on endogenous stem/progenitor cell conversion into β -cells, or α - and subsequently into β -cells [33, 34]. It is possible that the expression of hPRDX4 can accelerate the replication of pluripotent cells to repair and remodel islets.

In conclusion, insulin-secreting β -cells in the Tg islets, where hPRDX4 is specifically expressed, are more significantly protective against SHDS-induced insulinitis and apoptosis than those of the WT mice. Additionally, the Tg islets are more likely to activate the proliferation of β -cells. Basically, two different mechanisms of PRDX4 in the suppression of apoptosis have been proposed. One pathway is linked to the direct functions of PRDX4 by scavenging increased ROS/oxidative stress due to high glucose levels (DM). The other suggests indirect functions of PRDX4 by preventing inflammatory cell (in particular, T-cell) infiltrate and/or down-regulating various proinflammatory mediators/cytokines and receptors/ligands involved in cell survival and growth. These observations indicate that these Tg mice could become a useful animal model to study the relevance between oxidative stress and inflammation, and that PRDX4 could be a potential target for ameliorating T1DM. Furthermore, PRDX4 may be pathophysiologically relevant to protection against DM in humans.

Other Intriguing Topics for Future Studies by Using Our Tg Mice

Based on our recent data above with regard to PRDX4 in T1DM [1], and the previous PRDXs papers in atherosclerosis [11, 13, 14], we can easily hypothesize that PRDX4 should reduce the progression of atheromatous plaques. Indeed, atherosclerosis is a complex inflammatory disease, and disturbed blood flow and inflammatory cytokines with subsequent generation of ROS derived from macrophages, SMCs, and endothelial cells must significantly contribute to its pathogenesis [4–7]. To define the role of PRDX4 in hyperlipidemia-induced atherosclerosis as described before [35–38], we crossed Tg mice onto apolipoprotein E (apoE) knockout (apoE^{-/-}) mice and generated hPRDX4^{+/+}/apoE^{-/-} mice, which we fed a high cholesterol diet (HcD). Our preliminary data revealed that the hPRDX4^{+/+}/apoE^{-/-} mice showed more suppressed and stable atheromatous plaques, along with less T-lymphocyte infiltration, less necrosis, and a larger amount of collagen content, as compared with those of control (apoE^{-/-}) mice (data in submission to Antioxidants & Redox Signaling). Furthermore, the migrating SMCs in the more thickened fibrous cap of hPRDX4^{+/+}/apoE^{-/-} mice were significantly larger in number, associated with more repressed apoptosis and decreased pro-apoptotic factor expression, resulting in markedly

lower vulnerability index. These preliminary data indicate that PRDX4, especially expressed in the SMCs, should be not only an anti-atherogenic factor but also a protective one against vulnerable plaques. According to the immunohistochemical analysis of various tissues of non-treated Tg mice described above, medial SMCs in the aorta showed a specific, but somewhat weak, expression of hPRDX4 [1]. However, interestingly, the hPRDX4^{+/+}/apoE^{-/-} mice demonstrated that after being fed an HcD their atherosclerotic aortas had a significantly higher expression of hPRDX4. These findings suggest that the hyperlipidemia observed in this model is prone to oxidation to generate ROS in the atheromatous plaques, leading to increased peroxidation with activated anti-oxidative proteins including PRDX4. Taken together with the overexpression of hPRDX4 in the PRDX4^{+/+}/apoE^{-/-} mice, the endogenous mPRDX4 expression was also significantly higher than that in the apoE^{-/-} mice.

In conclusion, the vascular cells including SMCs in the atherosclerotic lesions of the hPRDX4^{+/+}/apoE^{-/-} mice are more significantly protective against apoptosis and inflammation than those of the controls, resulting in the suppression of atherosclerotic formation and the induction of stable plaques. The mechanisms of PRDX4 in the reduction of atherosclerosis should involve scavenging increased ROS/oxidative stress and inhibiting pro-apoptotic and/or possibly inflammatory signaling activation, particularly of pro-apoptotic gene expression and/or T-cell infiltrates, respectively. Concerning the broad spectrum of substrates of PRDX4, all the results obtained in our experiments could be attributable not only to the protection against oxidative stress-dependent or -independent atherosclerosis, but possibly also to the prevention against oxidative stress-induced type 2 DM (T2DM), which is considered to be a chronic inflammatory process as well. Actually, the hPRDX4^{+/+}/apoE^{-/-} mice after the HcD for 12 wk showed significantly more suppressed blood pressure and borderline (in)significantly less hypoinsulinemia than the apoE^{-/-} mice. One possibility is that the present HcD-induced hypercholesterolemia model might cause not only advanced atherosclerosis but also DM or metabolic syndrome. Despite that, since there have been no collected or significant data related to glucose metabolism in more detail, we are going to perform a follow-up study with an increased number of samples. Nevertheless, these present observations indicate that a specific accelerator of PRDX4 proves to be a potential therapeutic agent for ameliorating atherosclerotic progression by suppressing oxidative damage and pro-apoptotic or inflammatory factors throughout the course of the disease. PRDX4 should be pathophysiologically relevant to protection against atherosclerosis and subsequently potential metabolic syndrome in humans.

Finally, we plan to perform another *in vivo* model on a high fructose diet with or without streptozotocin injection between Tg and WT mice in order to evaluate the functions of PRDX4 in insulin resistance, as a pre-T2DM state, or nonalcoholic steatohepatitis. It is because adiponectin, an inflammatory-inducing cytokine secreted from adipocytes, has been reported to be centrally involved in the pathogenesis of insulin resistance/metabolic syndrome, which increases the risk of atherosclerosis and cardiovascular events [39]. Furthermore, it would also be very intriguing to further study the close relationships between PRDX4 and the inhibition of cancer progression.

Summary

We reviewed the critical roles of PRDX4 in inflammatory diseases, such as DM and atherosclerosis. Among the PRDX family, PRDX4 is the only known secretory form located in the extracellular space, and it exerts its protective function against oxidative damage by scavenging ROS, i.e., in the vascular vessels. More recently, we generated human PRDX4 transgenic mice (Tg), and for the first time established a SHDS-induced T1DM model on our Tg mice [1]. Our present data have demonstrated that SHDS-treated Tg mice, which overexpressed human PRDX4 in pancreatic islets, can protect pancreatic b-cells against injury (insulinitis) by suppressing increased ROS/oxidative stress and inflammatory signaling activation [1]. Furthermore, in order to define the role of PRDX4 in hyperlipidemia-induced atherosclerosis, we generated Tg and apoE^{-/-} (hPRDX4^{+/+}/apoE^{-/-}) mice. Compared to control mice (apoE^{-/-}), they showed the following: fewer atheromatous plaques, less T-lymphocyte infiltration, less necrosis (apoptosis), a larger number of SMCs, and a larger amount of collagen, resulting in thickened fibrous cap formation and stable plaques (data in submission to *Antioxidants & Redox Signaling*). All these findings indicate that Tg mice, including hPRDX4^{+/+}/apoE^{-/-} mice, could become a useful animal model to study the relevance of oxidative stress to inflammation, and that a specific accelerator of PRDX4 might prove to be a potential therapeutic agent for ameliorating various chronic inflammatory diseases.

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ペルオキシレドキシン4：炎症性疾患における重要な役割

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要 旨：ペルオキシレドキシン4(PRDX4)が, 様々な炎症性疾患において重要な役割を有していることについて詳述する. PRDXは抗酸化作用を有する一酵素群として知られ, 少なくとも6種類に及んでおり, 多くの生体内で広く発現していることが報告されている. 特にPRDX4は, 他のPRDXと発現型を異にし, 唯一の分泌型として発現しており, 細胞外領域において酸化ストレスからの組織傷害を防御する役割を有するとされる. 最近我々は, ヒトPRDX4(human PRDX4)のトランスジェニックマウス(Tg)を作製し, streptozotocinの単回大量投与誘発1型糖尿病モデルを確立した. Tg豚の特にラ氏島においてヒトPRDX4が高発現しており, 増加する酸化ストレスおよび炎症性サイトカインを伴った膵島炎が, PRDX4により特異的に抑制, 防御され生体内で保護的に機能していることを報告した. PRDX4は将来的に, 動脈硬化や糖尿病を含む様々な炎症性疾患に対する有効な治療薬の一つとして期待される.

キーワード：ペルオキシレドキシン4, PRDX4, 炎症性疾患, ヒトPRDX4トランスジェニックマウス.

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