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Review

Is the oxidative stress theory of aging dead?

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ABSTRACT

Currently, the oxidative stress (or free radical) theory of aging is the most popular explanation of how aging occurs at the molecular level. While data from studies in invertebrates (e.g., *C. elegans* and *Drosophila*) and rodents show a correlation between increased lifespan and resistance to oxidative stress (and in some cases reduced oxidative damage to macromolecules), direct evidence showing that alterations in oxidative damage/stress play a role in aging are limited to a few studies with transgenic *Drosophila* that overexpress antioxidant enzymes. Over the past eight years, our laboratory has conducted an exhaustive study on the effect of under- or overexpressing a large number and wide variety of genes coding for antioxidant enzymes. In this review, we present the survival data from these studies together. Because only one (the deletion of the *Sod1* gene) of the 18 genetic manipulations we studied had an effect on lifespan, our data calls into serious question the hypothesis that alterations in oxidative damage/stress play a role in the longevity of mice.

1. Introduction

The free radical theory of aging proposed in the 1950s by Denham Harman [1], postulates that oxygen free radicals formed endogenously from normal metabolic processes play a role in the aging process because of an increase in oxidative damage to macromolecules. The free radical theory of aging has since been modified to the oxidative stress theory of aging because oxygen species such as peroxides and aldehydes, which are not technically free radicals, also play a role in oxidative damage to cells. The imbalance between prooxidants and antioxidants leads to an accumulation of oxidative damage in a variety of macromolecules with age resulting in a progressive loss in functional cellular processes, leading to the aging phenotype [2].

Several lines of evidence support the oxidative stress theory of aging. First, the levels of oxidative damage to lipid, DNA, and protein have been reported to increase with age in a wide variety of tissues and animal models [3]. Second, studies with animal models showing increased longevity are consistent with the oxidative stress theory of aging; the longer-lived animals show reduced oxidative damage and/or increased resistance to oxidative stress. For example, early studies on caloric restriction, which is the first and most studied experimental manipulation shown to increase lifespan and retard aging, showed that oxidative damage to lipid, DNA, and protein was reduced in caloric restricted rodents compared to rodents fed ad libitum (for review, see [3]). Subsequently, caloric restricted mice also were shown to be more resistant to oxidative stress [2,4,5]. In the 1990s, investigators showed that mutations in the insulin/IGF-1 signaling pathways (*age-1*, *daf-2*, and *daf-16* mutants) increased the lifespan of *C. elegans* that was correlated with increased resistance to oxidative stress [6–8] and reduced oxidative damage [9,10]. More recently, several genetic mouse models of longevity have been reported, e.g., Ames and Snell dwarf mice, $p66^{sch-/-}$ mice, and $Igf1r^{+/-}$ female mice (for a review, see [11]), and the increased lifespan of these models has be correlated to increased resistance to oxidative stress of either the cells from these mice or from the whole animal [12,13]; $p66^{sch-/-}$ mice [14], and $Igf1r^{+/-}$ female mice [15]. Thus, the observation that the experimental manipulations that increase lifespan in invertebrates and rodents correlate to increased resistance to oxidative stress or reduced oxidative damage provides strong evidence in support of the oxidative stress theory of aging. However, all of the experimental manipulations that increase lifespan also alter processes other than oxidative stress/damage; therefore, the increase in longevity in these animal models could arise through another mechanism.

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A direct experimental test of the oxidative stress theory of aging is to alter the level of oxidative stress/damage and determine how these alterations affect lifespan. Using DNA recombinant technology, investigators over the past fifteen years have studied the effect of altering the expression of various components of the antioxidant defense system on lifespan; these studies are described in the Discussion. Below, we bring together all of the lifespan data that our group has conducted on transgenic/knockout mice with alterations in a wide variety of genes involved in the antioxidant defense system. These data demonstrate that almost all alterations in the antioxidant system of mice have no effect on lifespan.

2. Methods

2.1. Animals

All mice were maintained under pathogen-free barrier conditions using microisolator cages in a temperature controlled environment as previously described [16,17]. Mice were housed four per cage following weaning and fed ad libitum with commercial mouse chow (Teklad Diet LM485). The mice were genotyped at 4 to 5 weeks of age by PCR analysis of DNA obtained from tail clips. Mice were assigned to survival groups at 2 months of age and allowed to live out their entire lifespan, i.e., there was no censoring of the mice when measuring survival. All procedures followed the guidelines approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and South Texas Veterans Health Care System, Audie L. Murphy Division. A list and description of the genetic manipulations in the antioxidant defense system that were used in the studies described in this review are given in Table 1.

2.2. Analysis of lifespan

Mice in the survival groups were allowed to live out their life, and the lifespan for individual mice was determined by recording the age of spontaneous death. The survival curves were compared statistically using the log–rank test [18], and the median, mean, 90th percentile (when 90% of the mice died), and maximum survivals were calculated for each group. Mean survivals (±SEM) for each experimental group were compared to the respective wild type (WT) group by performing a Student's *t*-test upon log-transformed survival times. The median and 90th percentile survivals for each group were compared to the WT group using a score test adapted from Wang et al. [19]. All comparisons were made individually between each experimental group and the WT group, and in the case where multiple experimental groups were compared to one WT group, Holm's method [20] was used to correct for multiple comparisons.

3. Genetic manipulated mice models

3.1. Knockout mice

The first mouse model we studied was mice deficient in MnSOD (*Sod2*), which plays a major role in the detoxification of superoxide anions generated in the mitochondria. Mice lacking MnSOD die within days or weeks after birth from cardiomyopathy or neurodegeneration, depending on the genetic background [21–23]. $Sod2^{+/-}$ mice show reduced (~50%) MnSOD in all tissues studied [17] and both embryonic fibroblasts from these mice and the whole animals [17] are more sensitive to oxidative stress. Furthermore, tissues from these mice show a significant increase in oxidative damage to DNA[17]. As the data in Fig. 1A and Table 2 show, the $Sod2^{+/-}$ mice show no difference in lifespan relative to WT mice [17].

CuZnSOD (*Sod1*) is the major superoxide dismutase isozyme found in cells and is localized in the cytosol and the intermembrane space of the mitochondria [24,25]. Mice null for CuZnSOD are viable and appear normal at birth [26]. Studies by other laboratories have reported a number of moderate to more severe pathologies in the $Sod1^{-/-}$ mice [27–29]. For example, $Sod1^{-/-}$ females are almost totally infertile due to ovarian-dysfunction [27,30], and $Sod1^{-/-}$ mice show very high levels of oxidative stress in several tissues and plasma and an accelerated loss of hind limb muscle mass with age that is associated with a phenotype consistent with distal axonopathy [31]. In 2005, Huang's laboratory reported that $Sod1^{-/-}$ mice show a decrease in lifespan, approximately 30%, which is associated with a high incidence of hepatocellular carcinoma [32]. We observed a ~30% decrease in mean, median, and maximum lifespan of the $Sod1^{-/-}$ mice (Fig. 1B and Table 2). Neither Huang's laboratory [33] nor our laboratory found any decrease in the lifespan of the $Sod1^{+/-}$ mice compared to WT mice (unpublished data). Currently there is no information about the levels of oxidative stress/damage in the $Sod1^{+/-}$ animals.

Glutathione peroxidase 1 (Gpx1) is also viewed as one of the major cellular scavengers of hydrogen peroxide (H_2O_2) and alkyl hydroperoxides in the cell [34]. Mice null for Gpx1 are viable and have normal development [35]. However, they are highly sensitive to both paraquat and diquat [36,37] and show increased oxidative damage to DNA [38]. Mice null for Gpx1 develop a high incidence of cataracts at a young age [39], suggesting an accelerated aging phenotype. The data in Fig. 1C and Table 2 show that $Gpx1^{-/-}$ mice show no difference in lifespan compared to WT mice.

We studied the effect of glutathione peroxidase 4 (Gpx4) deficiency on lifespan because this enzyme plays a unique role in the detoxification of lipid peroxides in membranes. Gpx4 is widely expressed in tissues at low levels compared to Gpx1; it is found in the cytosol, mitochondrial and nuclear fractions [40]. Among all

Table 1
List of knockout/transgenic mice studied.

Gene	Description of genetic manipulation	Abbreviation	Reference
Knockout mice			
Mn-superoxide dismutase	Deletion of exon 3 of Sod2 gene	$Sod2^{+/-}$	[21]
Cu/Zn-superoxide dismutase	Deletion of exon 3 and 4 of Sod1 gene	$Sod1^{-/-}$	[32]
Glutathione peroxidase 1	Deletion of exon 2 of Gpx1 gene	$Gpx1^{-/-}$	[35]
Glutathione peroxidase 4	Deletion of exon 3, 4, 5, 6, and 7 of Gpx4 gene	$Gpx4^{+/-}$	[16]
Methionine sulfoxide reductase A	Deletion of exon 2 on MsrA gene	$MsrA^{+/-}$	[45]
Thioredoxin 2	Mutational insertion in exon 1 of Trx2 gene	$Trx2^{+/-}$	[48]
Transgenic mice			
Cu/Zn-superoxide dismutase	64 kb genomic fragment of the human SOD1 gene containing 27 kb of 5′ and 3′-flanking sequences.	SOD1 Tg	[53]
Mn-superoxide dismutase	13 kb of the genomic fragment of Sod2 mouse gene.	*Sod2* Tg	[55]
Catalase	80 kb genomic fragment of human catalase gene containing 41 kb of 5′ and 6 kb of the 3′flanking sequences.	CAT Tg	[53]
Glutathione peroxidase 4	53 kb genomic fragment of human Gpx4 gene containing 30 kb of 5′ and 20 kb of 3′ flanking sequences.	GPX4 Tg	[58]

The descriptions of the genetic manipulation for each animal model described in the table were taken from the references given.

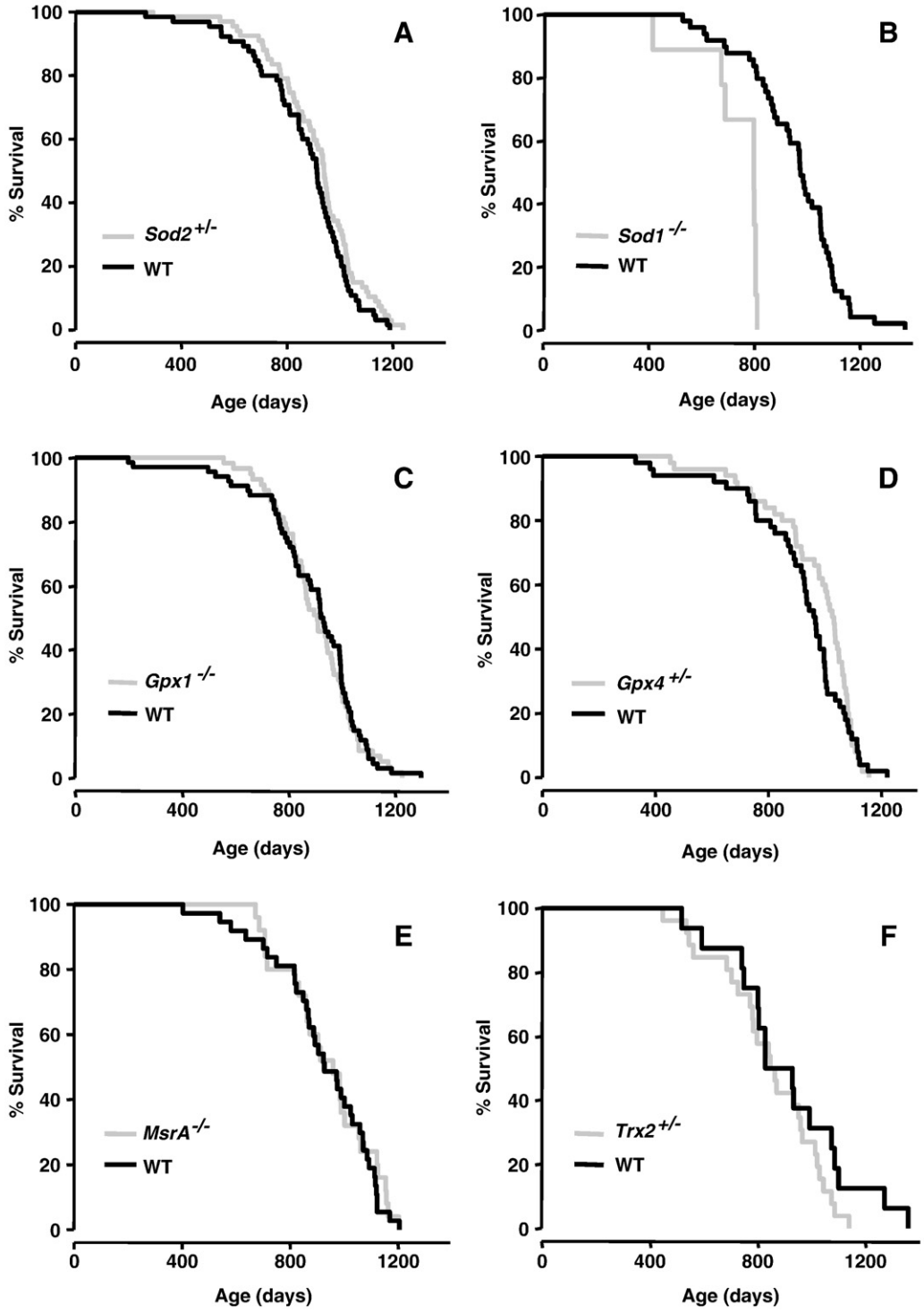

Fig. 1. Lifespans of knockout mice with a deficiency in various antioxidant enzymes. The survival curves of *Sod2*$^{+/-}$ (Graph A; [17]), *Sod1*$^{-/-}$ (Graph B; unpublished), *Gpx1*$^{-/-}$ (Graph C; [43], *Gpx4*$^{+/-}$ (Graph D; [16]), *MsrA*$^{-/-}$ (Graph E; [42], and *Trx2*$^{+/-}$ (Graph F; unpublished) mice are shown compared to their WT cohorts. The genetic background, number, sex and survival data for these curves are given in Table 2.

glutathione peroxidases, Gpx4 is the only peroxidase that can catalyze the reduction of complex lipid hydroperoxides, e.g., phospholipid hydroperoxides as well as hydroperoxides of cholesterol esters [41,42]. Null mice for Gpx4 are embryonic lethal (abnormal embryo development at stage E7.5; [43]). *Gpx4*$^{+/-}$ mice showed reduced Gpx4 protein levels and activity in the cytosolic and mitochondrial fractions from all tissues studied, and whole animals as well as embryonic fibroblasts from these mice were more sensitive to oxidative stress [43]. In addition, oxidative damage was increased in embryonic fibroblasts from the *Gpx4*$^{+/-}$ mice, as indicated by increased levels of F_2-isoprostanes and 8-oxo-2-deoxyguanosine in these cells [44]. Based on the oxidative stress theory, we predicted that the *Gpx4*$^{+/-}$ mice would show reduced lifespan because of the reduced ability to repair oxidative damage to membranes. However, we actually observed a slight (~7%), but significant, extension in median lifespan (Table 2), which appears to

Table 2
Survival data for mice deficient in antioxidant enzymes.

Genotype	Strain	Sex	*N*	Survival curve (p =)	Mean	Median	90%	Maximum	Reference
WT	C57BL/6	Female	66		894 ± 19	913 (855–943)	1034 (1002–1009)	1189	[17]
Sod2$^{+/-}$	C57BL/6	Female	68	0.12	918 ± 19	940 (900–959)	1088 (1044–1196)	1239	[17]
WT	C57BL/6	Female	50		915 ± 27	963 (923–996)	1145 (1071–1220)	1220	[16]
Gpx4$^{+/-}$	C57BL/6	Female	50	0.32	964 ± 23	1029 (979–1050)*	1026 (1085–1157)	1157	[16]
WT	C57BL/6	Mixed	68		891 ± 24	926 (874–994)	1091 (1040–1188)	1298	[38]
Gpx1$^{-/-}$	C57BL/6	Mixed	59	0.70	903 ± 19	908 (853–963)	1063 (1031–1183)	1226	[38]
Sod2$^{+/-}$ *X Gpx1*$^{+/-}$	C57BL/6	Mixed	25	0.61	880 ± 33	877 (791–943)	1057 (1027–1298)	1283	[38]
Sod2$^{+/-}$ *X Gpx1*$^{-/-}$	C57BL/6	Mixed	33	0.76	905 ± 25	911 (833–983)	1121 (1069–1248)	1248	[38]
Control[a]	Mixed[b]	Male	37		925 ± 32	926 (868–1030)	1120 (1092–1204)	1204	[46]
MsrA$^{-/-}$	Mixed[b]	Male	25	0.87	942 ± 34	959 (858–1056)	1156 (1122–1203)	1203	[46]
WT	C57BL/6	Mixed	50		902 ± 23	921 (874–993)	1076 (1035–1298)	1298	UP
Sod1$^{-/-}$	C57BL/6	Mixed	10	<0.001	693 ± 41*	755 (638–761)	762 (761–767)	767	UP
Gpx1$^{-/-}$XSod1$^{-/-}$	C57BL/6	Male	11	0.004	725 ± 48	773*(674–823)	828 (799–868)	868	UP
Gpx4$^{+/-}$XSod1$^{-/-}$	C57BL/6	Male	16	0.004	667 ± 42*	672*(563–817)	866 (817–883)	883	UP
Sod1$^{-/-}$XSod2$^{+/-}$	C57BL/6	Mixed	11	<0.001	730 ± 53	778 (674–823)	886(817–883)	908	UP
Gpx4$^{+/-}$X Gpx1$^{-/-}$	C57BL/6	Mixed	40	0.85	933 ± 25	917 (866–1013)	1124 (1086–1248)	1248	UP
Gpx4$^{+/-}$XSod2$^{+/-}$	C57BL/6	Mixed	11	0.85	906 ± 33	918 (824–1017)	1025 (938–1099)	1099	UP
WT	Mixed[b]	Female	16		913 ± 56	879 (802–1074)	1186 (1086–1359)	1359	UP
Trx2$^{+/-}$	Mixed[b]	Female	26	0.23	846 ± 3 6	855 (781–959)	1059 (1020–1139)	1139	UP

The survival data were taken from references given (UP = unpublished) and are presented together in the groups of mice in which the survival experiments were conducted. The survival data are expressed in days as mean ± SEM, median and 90% (when 90% of the mice have died) with 95% confidence interval in parenthesis, and maximum (age when the oldest mouse in the cohort died). The survival curves for the WT and knockout mice were statistically analyzed by the log–rank test, and the p values are given under survival curve column.

[a] Control mice contained both *MsrA*$^{+/+}$ and *MsrA*$^{+/-}$ mice.

[b] The genetic background is a mixture of C57BL/6 and 129.

* Values significantly different from the WT mice at the $p<0.05$ level.

be due to a delay in the incidence of cancer in the *Gpx4*$^{+/-}$ mice [16]; neither the survival curve (Fig. 1D), the mean, nor the 90% survival was significantly altered in the *Gx4*$^{+/-}$ mice.

Methionine sulfoxide reductase-A (MsrA) repairs oxidized methionine residues within proteins and also may function as a general antioxidant. In 2001, Moskovitz et al. reported that *MsrA*$^{-/-}$ mice have increased sensitivity to hyperoxia and show a major decrease (~40%) in lifespan [45]. We also observed that embryonic fibroblasts from *MsrA*$^{-/-}$ mice as well as whole animals showed increased sensitivity to oxidative stress [46]. However, as shown in Fig. 1E and Table 2, we did not observe any decrease in the lifespan of *MsrA*$^{-/-}$ mice. These contradictory data show the importance of replicating lifespan studies, and the possible reason for these contradictory data are presented in the Discussion.

Thioredoxin 2 (Trx2), which is the mitochondrial form of thioredoxin, is the electron donor for several antioxidant enzymes (perroxiredoxins, MsrA, etc.), but also plays a major role in repairing the oxidation of cysteine residues in proteins [47]. Previous studies demonstrated that Trx2 null mice are embryonic lethal at stage E 8.5, and the histological assessment of these embryos indicate a high incidence of apoptosis [48]. *Trx2*$^{+/-}$ mice were viable and showed reduced levels of Trx2 in all tissues studied. In addition, the *Trx2*$^{+/-}$ mice display diminished mitochondrial functions (decreased ATP synthesis and increased ROS production) in several tissues studied. Additionally, we found increased levels of oxidative damage to DNA, lipids and protein [49]. The *Trx2*$^{+/-}$ mice showed a slight decrease (7%) in mean lifespan and a 16% decrease in maximum lifespan (Fig. 1F and Table 2). However, the lifespan curves were not significantly different, and the decrease in mean, median, and 90% survival were not significantly different. This difference in lifespan might be significant with a greater sample size.

In addition to studying the effect of a reduction or complete deletion of a single antioxidant enzyme gene on lifespan, we also studied the effect of deletions in more than one antioxidant gene on lifespan, and these data are presented in Table 2. We hypothesized that reducing the expression of more than one antioxidant enzyme at a time, i.e., reducing two pathways in the antioxidant defense system, would affect longevity when reducing one pathway might not. In an attempt to test this hypothesis, we measured the lifespan of mice that have decreased levels of the following pairs of antioxidant enzymes: CuZnSOD and MnSOD (*Sod1*$^{-/-}$/*Sod2*$^{+/-}$); CuZnSOD and Gpx1 (*Sod1*$^{-/-}$/*Gpx1*$^{-/-}$); CuZnSOD and Gpx4 (*Sod1*$^{-/-}$/*Gpx4*$^{+/-}$); MnSOD and Gpx1 (*Sod2*$^{+/-}$ and *Gpx1*$^{-/-}$); MnSOD and Gpx4 (*Sod2*$^{+/-}$/*Gpx4*$^{+/-}$) and Gpx1 and Gpx4 (*Gpx1*$^{-/-}$/*Gpx4*$^{+/-}$). Only the double mutants that are null for *Sod1* show significant differences in the survival curves compared to WT mice (Table 2), and these mice show an ~20% decrease in mean lifespan and a more than 30% decrease in maximum lifespan. These data show that a lack of CuZnSOD consistently reduces lifespan no matter what changes occur in other antioxidant enzymes.

3.2. Transgenic mice

Overexpressing CuZnSOD has been shown to increase the lifespan of *Drosophila* [50–52]. CuZnSOD transgenic mice (SOD1 Tg) were generated using a large fragment of human genomic DNA containing the SOD1 gene (Table 1), and we studied the effect of overexpressing CuZnSOD on the lifespan of mice (Fig. 2A). We showed that the activity of CuZnSOD was two- to five-fold higher in tissues of the SOD1 Tg compared to WT mice [53]. Embryonic fibroblasts from the SOD1 Tg mice were more resistant to paraquat toxicity ([54] and Fig. 3). Moreover, data from whole animal indicate that SOD1Tg mice are more resistant to oxidative stress induced by paraquat, and levels of lipid peroxidation (measured as 8-isoprostane) induced by diquat treatment are lower in SOD1Tg mice compared to WT mice, as would be predicted from an increase in CuZnSOD expression (unpublished data). The data in Fig. 2A and Table 3 show that the lifespan of SOD1 Tg mice was not significantly different from the lifespan of WT mice. These data are in agreement with the previous study from Epstein's laboratory using a different transgenic mouse model in which Huang et al. [33] showed that the lifespan of transgenic mice overexpressing CuZnSOD (two- to five-fold increase) was similar to WT mice.

Catalase is an antioxidant enzyme found in all aerobic cells that catalyzes the decomposition of hydrogen peroxide to oxygen and water. Catalase transgenic (CAT Tg) mice were generated in our laboratory using a large genomic fragment of human DNA containing the catalase gene (Table 1). Catalase activity is two- to four-fold higher in the tissues of the CAT Tg mice and is expressed in the peroxisomes [53]. Embryonic fibroblasts from CAT Tg mice are more resistant to hydrogen peroxide toxicity [54]. Data obtained from whole animal

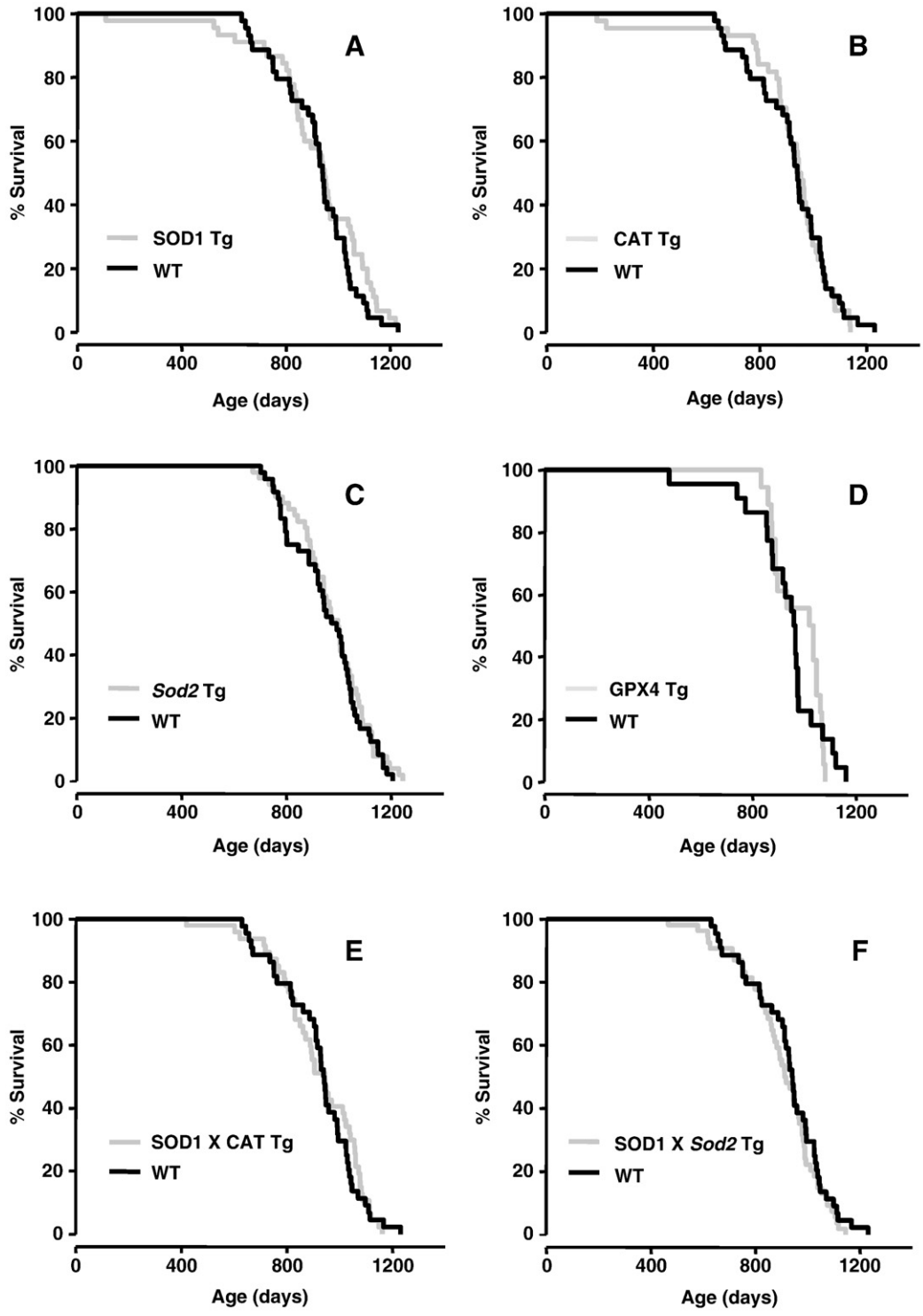

Fig. 2. Lifespans of transgenic mice overexpressing different antioxidant enzymes. The survival curves of SOD1 Tg (Graph A; [103], CAT Tg (Graph B; [103]), *Sod2* Tg (Graph C; [53]), GPX4 Tg (Graph D; unpublished), SOD1/CAT Tg (Graph E; [103]), and SOD1/*Sod2* Tg (Graph F; [103]) mice are shown compared to their WT cohorts. The genetic background, number, sex and survival data for these curves are given in Table 3.

indicate that CAT Tg mice have less DNA oxidation (measured by 8oxodG) in all the tissues studied compared to WT mice (unpublished data). As shown in Fig. 2B and Table 3, we observed no difference in lifespan of CAT Tg mice compared to WT mice.

In 2001, Epstein's laboratory generated transgenic mice overexpressing MnSOD (*Sod2* Tg) using a genomic fragment of the mouse *Sod2* gene (Table 1). These mice show a ~two-fold overexpression of MnSOD in all tissues examined [55]. Hu et al., reported that overexpression of MnSOD increased the maximum lifespan of transgenic mice using a transgene with the *Sod2* cDNA fused to β-actin promoter (two- to four-fold increase, except liver) [56]. We showed that embryonic fibroblasts from the *Sod2* Tg mice are more resistant to paraquat (Fig. 3C) and that oxidative damage (protein carbonyls and F_2-isoprostanes) is attenuated in old *Sod2* Tg mice [57]. However, as shown in Fig. 2C and Table 3, no difference was observed in the lifespans of the *Sod2*-Tg and WT mice.

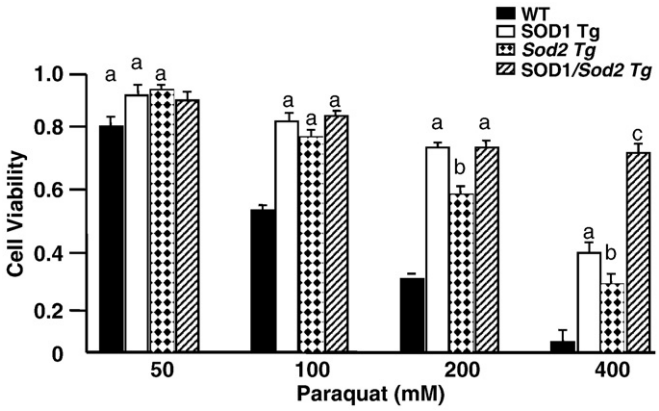

Fig. 3. Sensitivity of transgenic mice to oxidative stress. The sensitivity of embryonic fibroblasts isolated from WT, SOD1 Tg, *Sod2* Tg, and SOD1/*Sod2* Tg mice to paraquat (48 h) was determined as previously described [59]. The data are the mean ± SEM of experiments repeated with fibroblasts derived from three animals and were analyzed by two-way ANOVA. Values that are significantly different ($p<0.05$) from each other are shown with different subscripts.

In 2004, we generated Gpx4 transgenic mice (GPX4 Tg) using a large fragment of human genomic DNA containing the GPX4 gene (Table 1). Expression of the Gpx4 protein is two- to three-fold higher than in the GPX4 Tg mice compared to WT mice. Embryonic fibroblasts from the GPX4 Tg mice were more resistant to *t*-butylhydroperoxide and diquat, and diquat-induced liver damage and lipid peroxidation were significantly reduced in vivo in the GPX4 Tg mice [58]. In addition, diquat-induced caspase 3 activation and cytochrome c release from the mitochondria were significantly reduced in GPX4 Tg mice showing that overexpressing Gpx4 protected cells from oxidative stress induced apoptosis [58]. The data in Fig. 2D and Table 3 show that the lifespan of GPX4 Tg mice was not significantly different from WT mice.

We also have determined the effect of overexpressing multiple antioxidant genes, hypothesizing that antioxidant enzymes might work synergistically. First, we studied mice overexpressing both CuZnSOD and catalse (SOD1/CAT Tg), predicting that the increased hydrogen peroxide generated by overexpressing CuZnSOD would be converted to water by overexpressing catalase. Embryonic fibroblasts from SOD1/CAT Tg mice were resistant to both paraquat and hydrogen peroxide while embryonic fibroblasts from either SOD1 Tg or CAT Tg mice were resistant to only paraquat or hydrogen peroxide, respectively [54,59]. The data in Fig. 2E and Table 3 show that the lifespan of the SOD1/CAT Tg was essentially the same as that of WT, SOD1 Tg, or CAT Tg mice. In other words, there was no benefit of simultaneously overexpressing both CuZnSOD and catalase on lifespan. Second, we studied the effect of simultaneously overexpressing CuZnSOD and MnSOD (SOD1/*Sod2* Tg mice) on lifespan because SOD1/*Sod2* Tg mice would be predicted to have enhanced detoxification of superoxide anions in both the cytosol and mitochondria. The data in Fig. 3 show that embryonic fibroblasts from SOD1/*Sod2* Tg mice were more resistant to paraquat citotoxicity than embryonic fibroblasts from either SOD1 Tg or *Sod1* Tg mice. As shown in Fig. 2F and Table 3, the lifespan of SOD1/*Sod2* Tg mice transgenic was not significantly different than the lifespan of WT, SOD1 Tg, or *Sod2* Tg mice. Thus, simultaneous overexpression of superoxide dismutase in both the cytosol and mitochondria does not have a beneficial effect on longevity.

4. Discussion

The oxidative stress theory of aging has become the predominant theory to explain aging at the molecular level. Although there is a large amount of research over the past five decades supporting this theory, almost all the research has been correlative, e.g., a correlation between increased oxidative damage and age and a correlation between manipulations that increase lifespan and a reduction in oxidative damage and/or increase in resistance to oxidative stress. However, direct evidence showing that oxidative damage/stress alters aging was limited until the advent of genetic technology allowing investigators to alter the expression of antioxidant enzymes, which, because of their role in detoxification of free radicals and reactive oxygen species, can alter the sensitivity of the organism to oxidative stress and the levels of oxidative damage in cells and tissues.

Longevity or lifespan is the most acceptable parameter that has been used for several years to study aging, for example, crucial data obtained in several animal models with genetic mutations e.g., *C. elegans (age-1, daf-2, daf-16* mutants), yeast *(sir2), Drosophila* (*methusela* and its ligand, *stunted*), and mice (Ames dwarf mice), etc. have used lifespan as determinant of aging. Ideally, it would be better to determine other parameters involved in changes in the basic mechanisms of aging or healthspan. However, nowadays there is no consensus about how to define healthspan and how to measure this parameter in all of these model systems. Nevertheless, we do know that it is possible to retard aging in multiple animal models and simultaneously lengthen lifespan; for example, when mice and rats are fed restricted amounts of food, aging mechanisms appear to be delayed and the animals live longer. It is possible that the genetic alterations being studied may have slowed the rate of aging in certain tissues even though the mice were not living longer; however, the pathology obtained from some of these lines showed no major differences between mice genetically manipulated and WT mice, with the exception of MnSOD heterozygous mice, where this deficiency in MnSOD in mice resulted in a significant increase in tumor incidence and without any difference in lifespan observed [17].

In this review article, we present data that we have generated over the past eight years on the lifespan of mice with alterations in various enzymes in the antioxidant defense system. When using lifespan to determine whether an experimental manipulation alters aging, it is critical that lifespan be determined under optimal husbandry conditions

Table 3
Survival data for mice overexpressing antioxidant enzymes.

Genotype	Strain	Sex	N	Survival curve (p =)	Mean	Median	90%	Maximum	Reference
WT	C57BL/6	Male	47		982 ± 20	960 (920–1028)	1128 (1080–1206)	1206	[57]
Sod2 Tg	C57BL/6	Male	50	0.48	997 ± 12	977 (943–1035)	1165 (1092–1245)	1245	[57]
WT	C57BL/6	Male	22		933 ± 31	963 (879–975)	1106 (1026–1161)	1161	UP
Gpx4 Tg	C57BL/6	Male	18	0.84	977 ± 21	1028 (888–1062)	1072 (1062–1080)	1080	UP
WT	C57BL/6	Male	44		922 ± 22	941 (911–991)	1090 (1038–1235)	1231	[103]
SOD1 Tg	C57BL/6	Male	45	0.27	915 ± 40	944 (859–1038)	1131 (1111–2120)	1229	[103]
CAT Tg	C57BL/6	Male	44	0.87	916 ± 28	949 (904–978)	1099 (1036–1139)	1139	[103]
SOD1 Tg X CAT Tg	C57BL/6	Male	47	0.75	922 ± 23	945 (863–1025)	1098 (1078–1163)	1163	[103]
SOD1 Tg X *Sod2* Tg	C57BL/6	Male	54	0.41	899 ± 20	914 (868–967)	1075 (1038–1117)	1144	[103]

The survival data were taken from references (UP = unpublished) given and are presented together in the groups of mice in which the survival experiments were conducted. The survival data are expressed in days as mean ± SEM, median and 90% (when 90% of the mice have died) with 95% confidence interval in parenthesis, and maximum (age when the oldest mouse in the cohort died). The survival curves for the WT and transgenic mice were statistically analyzed by the log–rank test, and the *p* values are given under survival curve column.

to eliminate/minimize deaths from non-aging causes, e.g., infectious disease, inflammation, stress, etc. This is particularly important for studies using genetic manipulations in the antioxidant defense system because these genetic manipulations have the potential of altering survival when animals are maintained under sub-optimal husbandry conditions where the animals are exposed to increased stress/inflammation. The husbandry conditions used in our studies were optimal as shown by the long lifespans of the WT mice used in these studies, e.g., the mean and maximum survivals of more than 30 and 40 months, respectively, which is as long if not longer than the lifespans of similar inbred mice reported by other laboratories, including the aging colony maintained by the NIA [11,16]. For example, data published by the Jackson Laboratory show that C57B6 mice have mean and maximal lifespans of 27 and 40 months, respectively (mean lifespan approximately 3 months shorter compared to our data) [60,61]. However, the comparisons of others parameters such as food intake, body weight, fecundity and incidence of tumors, are similar to the data coming from our animal facility [60–62]. Therefore, altogether the data indicate that our husbandry has optimal healthy conditions that allow us to observe an extension in the lifespan of mice without changes in other parameters, such as body weight and food consumption.

Thus, the lifespan data we have generated in our studies allow us to determine with a high degree of accuracy whether the genetic manipulations in various components of the antioxidant defense system alter mouse longevity.

In the first series of experiments, we studied the effect of targeted genetic deletions in antioxidant enzymes on lifespan with the prediction that mice deficient or lacking one or more antioxidant enzymes would show an increase in the sensitivity of cells/tissues to oxidative stress resulting in increased oxidative damage and reduced lifespan. Previous studies with invertebrates have given mixed results with respect to the effect of reducing antioxidant gene expression on lifespan. In yeast, both clonal and chronological lifespan was shortened by deleting either CuZnSOD [63,64], MnSOD [64,65], MsrA, or MsrB [66]. However, under anaerobic conditions, deletion or overexpression of MsrA or MsrB had no effect on clonal lifespan [66]. In *C. elegans,* Doonan et al. [67], Yang et al. [68], Yen et al. [69], and Van Raamsdonk and Hekimi [70] demonstrated that the absence of SOD genes (both the cytosolic and mitochondrial isoforms) had no effect on lifespan, either in WT or in long-lived mutants despite the fact that these genetic manipulations increased both the sensitivity of the nematodes to oxidative stress (paraquat) and levels of oxidative damage to proteins. *Drosophila* lacking either CuZnSOD (*SOD1*) or MnSOD (*SOD2*) show a severe phenotype, e.g., deletion in CuZnSOD resulted in 80% reduction in lifespan [71,72]; deletion in Sod2 induced postnatal lethality [73,74]; *Drosophila* heterozygous for the *SOD1* gene show no significant differences, and *SOD2* gene show a slight reduction in lifespan [71,74,75].

In mice, two previous studies have measured the lifespan of mice lacking an antioxidant enzyme. In 2001, Moskovitz et al. [45] reported an ~40% decrease in mean and maximum lifespans in $MsrA^{-/-}$ mice, and in 2005, Elchuri et al. [32] reported that $Sod1^{-/-}$ mice have a 30% reduction in mean lifespan and an ~40% in maximum lifespan. We have replicated the study by Elchuri et al. [32] showing that the mean, median, 90%, and maximum lifespans of $Sod1^{-/-}$ mice are reduced 20% to 40% compared to WT mice. However, we were unable to replicate the study by Moskovitz et al. [45]; we found no difference in the lifespans of $MsrA^{-/-}$ and WT/$MsrA^{+/-}$ mice. The difference in lifespan observed in the two studies is not due to the genetic background of the mice because the mice used in both studies were a mixture of C57BL/6 and 129. Rather, the small sample size and sub-optimum conditions used in the study by Moskovitz et al. [45] are a more likely cause of this discrepancy. Our larger sample size lessens the influence that each animal has on the overall survival, i.e., the survival data are less likely to be distorted by outliers that arise from maternal- or paternal-specific effects on lifespan [11,76]. Moskovitz et al. used the following number of mice in their study: 17 $MsrA^{-/-}$, 8 $MsrA^{+/-}$, and 14 WT mice. However, the most likely reason for the discrepancy between our studies is that the mice in used in the study by Moskovitz et al. [45] appear to have been maintained under sub-optimum conditions, i.e., the mice were relatively short-lived. The mean lifespan of WT mice in the study by Moskovitz et al. was 680 days compared to the mean lifespan of 925 days for WT mice in our study. In other words, the husbandry conditions used in our study resulted in a ~35% longer lifespan. Thus, the reduction in lifespan of $MsrA^{-/-}$ mice observed by Moskovitz et al. could be due to the environment under which the mice were studied rather than due to accelerated aging because no difference in lifespan is observed when mice are maintained under conditions where they are able to live out their entire lifespan.

We also studied the effect of reduced expression of other antioxidant enzymes on lifespan, e.g., $Sod2^{+/-}$, $Gpx1^{-/-}$, $Gpx4^{+/-}$, and $Trx2^{+/-}$ mice, and found no significant differences in the lifespans of these mice compared with their WT littermates. In fact, we found that reduced expression of Gpx4 resulted in a slight but significant increase in the median lifespan [16]. Furthermore, we studied the effect of reduced expression of various combinations of antioxidant enzymes on lifespan and found that only those mice lacking CuZnSOD showed a decreased in lifespan, which was similar to the lifespan for $Sod1^{-/-}$ mice. Therefore, our studies with knockout mice demonstrate that only mice that lack CuZnSOD show a reduction in lifespan. Although these mice exhibit some phenotypes of accelerating aging, i.e., increased age-related hearing loss [77–79], macular degeneration [80], early incidence of cataracts [81], vascular hypertrophy [82,83], and increased age-related muscle atrophy [31], they also had high incidence of hepatocellular carcinoma [32], which is never observed in C57BL/6 mice [62,84]. Therefore, it is uncertain whether the reduced lifespan observed in the $Sod1^{-/-}$ mice is due to accelerated aging or is from novel pathology arising from the genetic mutation.

Why do $Sod1^{-/-}$ mice show reduced lifespan while all the other mice we studied that were deficient in other antioxidant enzymes show no effect on lifespan? All the knockout mouse models we studied show increased sensitivity to oxidative stress, i.e., they exhibit the phenotype expected from the reduced expression of the antioxidant enzyme(s). However, the $Sod1^{-/-}$ mice appear to have greater endogenous stress as observed by oxidative damage and changes in gene expression. For example, we showed that the genetic expression profile in the livers of $Sod1^{-/-}$ mice differed from the profiles observed in WT or $Gpx1^{-/-}$ mice and showed that many of the changes in gene expression were similar to those found in the livers of WT or $Gpx1^{-/-}$ mice after induction of oxidative stress by diquat [37]. As shown in Fig. 4, DNA oxidation is four- to five-fold higher in the livers of $Sod1^{-/-}$ mice than WT mice; DNA oxidation is ~40% higher in the $Gpx1^{-/-}$ or $Sod2^{+/-}$ mice compared to WT mice. In other words, the level of DNA oxidation in the $Sod1^{-/-}$ mice is two-fold higher than that observed in the $Gpx1^{-/-}$ or $Sod2^{+/-}$ mice. It should be noted that, the levels of oxidative damage observed in the $Sod1^{-/-}$ mice are much greater than the normally observed in tissues of old mice. For example, we have observed that oxidative damage to DNA increases with age ~40% in the livers of mice [17,85], which is one-third that observed in the $Sod1^{-/-}$ mice but is similar to the increase in DNA oxidation observed in the $Gpx1^{-/-}$ or $Sod2^{+/-}$ mice. Thus, while $Sod1^{-/-}$ mice show an increase in oxidative damage and a decrease in lifespan, as would be predicted by the oxidative stress theory of aging, the levels of oxidative damage in the $Sod1^{-/-}$ mice are much higher than that observed even in old mice. In contrast, the levels of oxidative damage in $Sod2^{+/-}$ [17] and $Gpx1^{-/-}$ [43] mice are similar to those observed in old WT mice; however, and the lifespan of the $Gpx1^{-/-}$ and $Sod2^{+/-}$ mice are essentially identical to WT mice.

One of the problems in determining whether oxidative stress plays a role in aging using knockout mice to accelerate aging is that many

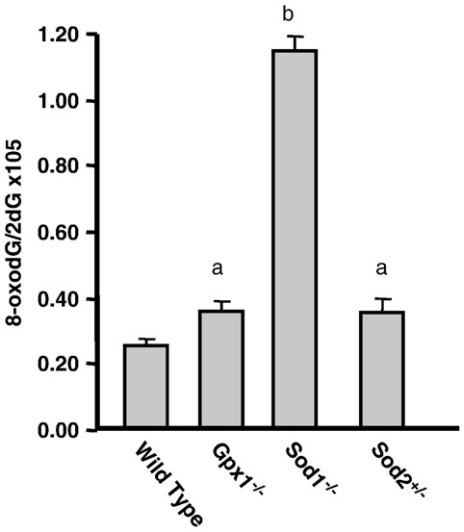

Fig. 4. Oxidative damage to macromolecules in knockouts mice. The levels of DNA oxidation measured as 8-oxodG/2dG in livers from young WT, Gpx1$^{-/-}$, Sod1$^{-/-}$, and Sod2$^{+/-}$ mice was determined as described previously [85]. The data are expressed as the mean ± SEM from 3 to 5 mice and were analyzed by the non-parametric test of ANOVA. Values that are significantly different ($p<0.05$) from each other are shown with different subscripts.

manipulations can shorten lifespan that would not have any effect on aging. For example, peroxiredoxin-1 (*Prdx*1) knockout mice (B6), are shown to have a significantly shortened lifespan; however, this effect is due mainly to a higher incidence in cancer burden (osteosarcoma, fibrosarcoma) and hemolytic anemia, which are not pathologies found in this strain of mice [86]. Therefore, most gerontologists agree that a manipulation that increases lifespan gives the greatest insight in to the mechanism of aging. In other words, determining whether an increase in the antioxidant defense system would increase lifespan would be more powerful evidence for oxidative stress/free radicals playing a role in aging than showing that a reduction in the antioxidant system decreases lifespan.

The effect of overexpressing antioxidant enzymes on lifespan in invertebrates has been mixed. The lifespan (clonal and chronological) of yeast has been reported to be increased by the overexpression of MsrA [66] and MsrB [66], and MnSOD [65,87]. Initial studies in *Drosophila* using P-element mediated transformation reported that overexpressing either CuZnSOD [72,88,89] or catalase [90] had no effect on lifespan. Later, Orr and Sohal [89] reported that overexpression of both CuZnSOD and catalase significantly increased the lifespan of *Drosophila*. However, the site of the P-element insertion can alter lifespan independently [91], and in a subsequent study with a large number of transgenic lines of *Drosophila*, Orr et al. [92] found that neither the overexpression of CuZnSOD and catalase, MnSOD, nor thioredoxin reductase significantly altered the lifespans of long-lived *Drosophila* strains. Using inducible systems to overexpress antioxidant genes to avoid the problems associated with the site of transgene insertion, Parkes et al. [51] and Sun and Tower [93] reported that overexpressing CuZnSOD increased the lifespan of *Drosophila*. Sun and Tower also reported that overexpression of catalase had no effect on lifespan and that there was no added benefit of overexpressing both catalase and CuZnSOD [93]. Subsequently, Sun et al. [94] reported that overexpression of MnSOD also increased the lifespan of *Drosophila* and that the simultaneous overexpression of MnSOD and CuZnSOD had an additional increase in lifespan effect [95]. Using 10 different genetic backgrounds of *Drosophila* of both sexes, Promislow's laboratory [96] reported that overexpression of CuZnSOD in motorneurones increased lifespan of long-lived flies; however, effect of CuZnSOD overexpression varied considerably with different genetic backgrounds. The effect of overexpressing CuZnSOD on lifespan was sex dependent, e.g., an increase in lifespan was observed in six of the strains of female *Drosophila*, but only one strain of male *Drosophila* showed an increase in lifespan [96]. The overexpression of MsrA has also been reported to increase the lifespan of *Drosophila* [97–99].

Our data show that transgenic mice overexpressing CuZnSOD, catalase, MnSOD, or Gpx4 have lifespans similar to WT mice even though cells/tissues from these mice show increased resistance to oxidative stress. Our data with SOD1 Tg mice confirm the previous study by Huang et al. [33], which used a different transgenic mouse model overexpressing CuZnSOD in a different strain of mice. Schriner et al. [100] previously reported that transgenic mice overexpressing catalase showed an ~21% increase in lifespan; however, in this study catalase overexpression was targeted to the mitochondria while in our study, catalase overexpression occurred in the peroxisomes [53], where catalase is normally expressed [101]. Moreover, data from the same group also showed that the cytosolic expression of catalase had no effect on lifespan [100]. Hu et al. [56] reported that transgenic mice overexpressing MnSOD showed an 18% increase in maximum lifespan (1095 days vs 1290 days); however, the mean survival of the transgenic mice overexpressing MnSOD was only 4% longer than the WT mice. However, Hu et al. [56] presented no statistical analysis of the survival data. We observed no statistical difference in the survival curves or in the mean or median survival. The maximum survival of the *Sod2*-Tg mice in our study was 3% longer than WT mice; however, to statistically assess whether changes in maximum lifespan are significant, it is necessary to compare survival ratios at some quantile where an adequate number of animals are still alive. The 90th percentile (when 90% of the mice have died and only 10% remain) is used for this purpose. We found no significant difference in the 90% survival between the *Sod2*-Tg and WT mice in our study. We also found that overexpressing both CuZnSOD and catalase or CuZnSOD and MnSOD had no effect on lifespan even though cells from the SOD1/CAT Tg and SOD1/*Sod2*-Tg mice showed greater resistance to oxidative stress than increased expression of one of the antioxidant enzymes.

In summary, our research with 18 different genetic manipulations in the antioxidant defense system show that only the mouse model null for *Sod1* had an effect on lifespan that would be predicted from the oxidative stress theory of aging. One could argue that we failed to observe an effect on lifespan because the cells/tissues of the knockout/transgenic mice up- or down-regulate other components of the antioxidant defense system that counter the reduced or increased expression of the specific antioxidant enzyme(s). Except for the *Sod1*$^{-/-}$ mice [32], we have no evidence that any of the other manipulations showed an alteration in any of the other major antioxidant enzymes [16,17,38,49,54,57–59,102]. However, one can always argue that some minor component of the antioxidant enzyme system is altered in response to the changes in the genetic manipulation. To our knowledge, the only detailed study of gene expression in mice with an alteration in the antioxidant defense system was conducted by our group for *Sod1*$^{-/-}$ and *Gpx1*$^{-/-}$ mice [37]. We showed that neither knockout mouse model resulted in an up-regulation of any classical antioxidant genes in liver; however, the *Sod1*$^{-/-}$ mice showed an up-regulation of thiol antioxidants (e.g., metallothione, glutathione, thioredoxin, sulfiredoxin, etc.). Interestingly, the Sod1 null mice showed the reduction in lifespan even though these genes were up-regulated. The strongest evidence that the knockout/transgenic mouse models we studied exhibit the phenotype predicted from the genetic manipulation is that cells from these mouse models show alterations in sensitivity to oxidative stress. Therefore, we believe that the fact that the lifespan was not altered in almost all of the knockout/transgenic mice is strong evidence against oxidative stress/damage playing a major role in the molecular mechanism of aging in mice.

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