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Research Paper

Effects of transgenic methionine sulfoxide reductase A (MsrA) expression on lifespan and age-dependent changes in metabolic function in mice

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ABSTRACT

Mechanisms that preserve and maintain the cellular proteome are associated with long life and healthy aging. Oxidative damage is a significant contributor to perturbation of proteostasis and is dealt with by the cell through regulation of antioxidants, protein degradation, and repair of oxidized amino acids. Methionine sulfoxide reductase A (MsrA) repairs oxidation of free- and protein-bound methionine residues through enzymatic reduction and is found in both the cytosol and the mitochondria. Previous studies in *Drosophila* have shown that increasing expression of MsrA can extend longevity. Here we test the effects of increasing MsrA on longevity and healthy aging in two transgenic mouse models. We show that elevated expression of MsrA targeted specifically to the cytosol reduces the rate of age-related death in female mice when assessed by Gompertz analysis. However, neither cytosolic nor mitochondrial MsrA overexpression extends lifespan when measured by log-rank analysis. In mice with MsrA overexpression targeted to the mitochondria, we see evidence for improved insulin sensitivity in aged female mice. With these and our previous data, we conclude that the increasing MsrA expression in mice has differential effects on aging and healthy aging that are dependent on the target of its subcellular localization.

1. Introduction

There is growing evidence that maintenance of cellular proteostasis is a significant determinant of both longevity and healthy aging across the animal kingdom [1,2]. In this regard, the cellular response to oxidative stress is of importance because oxidative damage to cellular proteins can compromise protein function leading to cellular dysfunction, pathological manifestation of disease, and mortality [3]. While much of the focus on cellular proteostasis has highlighted removal of damaged proteins through the ubiquitin-proteasome system or autophagy, some forms of protein oxidative damage can be eliminated by endogenous cellular repair systems *in vivo*. Repairing or refolding damaged proteins might be viewed as an energetically favorable means of proteostasis to the cell rather than complete destruction and replacement of damaged proteins [2]. Of the small group of known endogenous protein repair mechanisms, the methionine sulfoxide reductases (Msr) can uniquely repair oxidation of free- and protein-bound methionine residues through enzymatic reduction [4,5]. Beyond direct methionine oxidation repair, Msr may also more broadly protect proteins by acting as a part of a free-radical sink wherein easily oxidized (and subsequently reduced by Msr) methionine residues act as targets of oxidative stress and thereby protect other amino acids from damage [6].

In eukaryotes, Msr exist as two main isoforms, MsrA and MsrB (which are further divided into 3 subclasses in mammals), each with specificity to different epimers of methionine oxidation. Because it was discovered prior to MsrB, the majority of studies regarding the biological significance of Msr activity have focused on the MsrA isoform. In terms of aging, both MsrA and MsrB1 now appear to be dispensable for mouse longevity [7,8], though there was historical

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suggestion that mice lacking MsrA are short-lived [9]. However, MsrA has been reported to be required for longevity extension associated with either dietary restriction or genetic modulation of the *daf-2* gene in *C. elegans* [10,11]. On the other hand, increasing levels of MsrA, but not of MsrB, has been shown to significantly increase lifespan in *Drosophila* [12–14]. One key difference between these isoforms is that MsrA is found in both the cytosol and the mitochondria of mammalian cells, whereas MsrB subclasses have discrete subcellular locations [15]. Both mitochondrial and cytosolic forms of MsrA are translated from a single gene with localization being dependent on alternative translation initiation sites [16].

The generation of the first transgenic mouse models designed to express increased levels of MsrA was reported in 2010 [17]. We and others have found that increasing MsrA expression in mice can be protective in mouse disease models, but that this protection is dependent on the subcellular location to which increased MsrA expression is targeted. Increased levels of MsrA in its native myristoylated form in the cytosol protect against cardiac ischemia/reperfusion injury in a mouse Langendorff model [18]. In contrast, overexpression of MsrA in the mitochondria has no effect on this cardiac injury. Conversely, increasing MsrA in the mitochondria, but not the cytosol, prevents insulin resistance in mice fed a high fat/high sugar diet [19]. This metabolic benefit under metabolic stress is attributed, at least in part, to a shift toward preferential use of fatty acid oxidation as energy substrates in mice with mitochondria-targeted overexpression of MsrA.

With our previous data showing that increasing levels and/or activity of MsrA may be beneficial for some aspects of healthy aging in mice as well as reports that overexpressing MsrA can increase *Drosophila* lifespan [12,14], our main goal in this study was to determine the effect of increasing MsrA expression on mouse lifespan. Because MsrA is natively found in both cytosol and mitochondria in mammals, our study used two novel MsrA transgenic mouse models designed to target increased MsrA expression to either the cytosol or the mitochondria. This allowed us to determine not only whether overexpressing MsrA in mice would increase lifespan or slow aging but also to determine whether any differences were driven by the subcellular localization of this enzyme.

2. Results

To determine the effect of high levels of MsrA on mammalian aging, we assessed the lifespan of mice with MsrA overexpression targeted to either the cytosol (TgCyto MsrA) or to the mitochondria (TgMito MsrA). The generation and general characterization of these mice has been previously reported [17–19]. Mice for this study were produced using a hemizygous breeding scheme resulting in both transgenic and wild-type (control) littermates. TgCyto MsrA and TgMito MsrA mice were maintained as independent breeding lines and for assessment of longevity with control mice from each line being pooled for comparison. Although the Log Rank analysis of survival allows one to determine if an experimental manipulation affects the overall survival curve of a group of animals, the Gompertz analysis allows one to study more in depth the effect of a manipulation on the survival of a population because Gompertz pointed out that the rate of mortality increases exponentially with age [20]. This exponential increase occurs after an initial period of age-independent mortality pointed out by Makeham (*e.g.* neonatal deaths and childhood deaths due to infectious diseases), and so we have the Gompertz-Makeham Law, often referred to as a Gompertz analysis. When mortality is plotted on a semi-logarithmic scale, the x-intercept, or the initial hazard rate at time zero, gives an estimate of the age-independent mortality while the slope gives an estimate of the rate of aging (Fig. 1). Using this means of analysis, we found that the slope of the TgCyto MsrA mice is significantly smaller than that of the control (p=0.009) but not different from TgMito MsrA mice (p=0.07). Thus, TgCyto MsrA mice exhibit a decreased rate of aging compared to control mice. In contrast, TgMito MsrA mice do not significantly differ from TgCyto MsrA mice or control mice. Interestingly, TgCyto MsrA mice have a significantly higher x-intercept than control mice (p=0.02), but not higher than that of TgMito MsrA mice (p=0.07). A possible interpretation of this finding is that TgCyto MsrA mice have a larger early age-independent mortality, but those mice that survive the early period age at a slower rate than control mice. In contrast, TgMito MsrA mice do not significantly differ from control or TgCyto MsrA mice in either parameter.

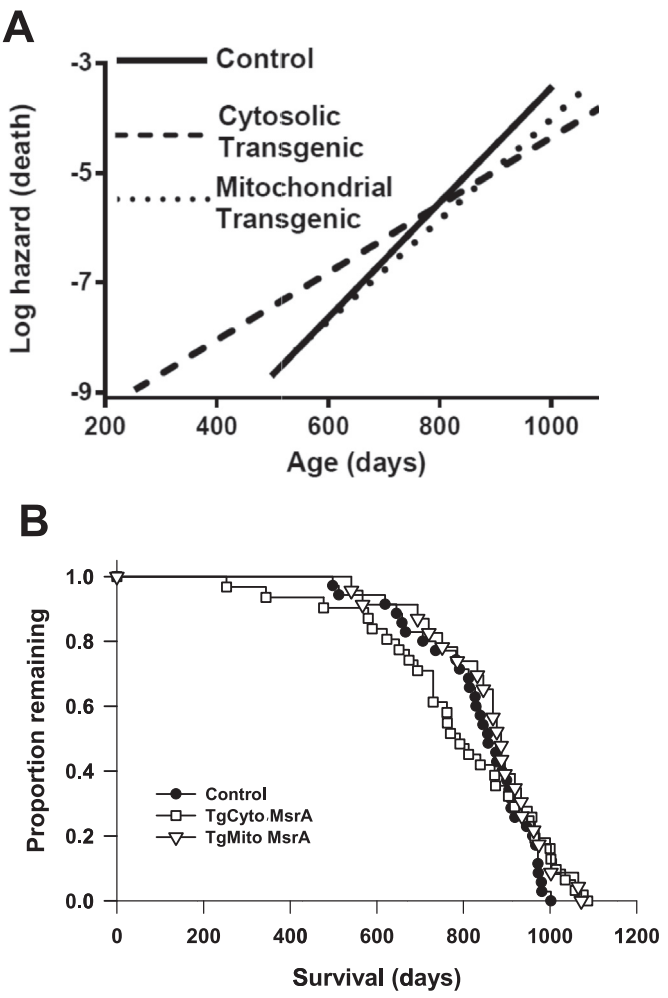

Fig. 1. Increased MsrA in the cytosol slows the rate of aging A. Gompertz slopes calculated across lifespan for Control (solid line), TgCyto MsrA (dashed line) and TgMito MsrA (dotted line). For each line, (slope, x-intercept) are as follows: control (0.01, −13.93), TgCyto MsrA (0.0062, −10.51), TgMito MsrA (0.0092, −13.22). B. Log Rank survival curve for Control (closed circle), TgCyto MsrA (open square) and TgMito MsrA (open triangle) female mice. Each symbol represents the natural death of an individual mouse.

We also show in Fig. 1 the survival curves of all three lines of mice. These data show that increasing levels of MsrA in either the cytosol or mitochondria had no significant effect on lifespan of female mice when assessed by either the standard log-rank test (p=0.52) or by Gehan-Breslow test (p=0.37) which gives more weight to earlier deaths. Mean, median and maximum (90%) survival data are presented in Table 1.

Table 1
Longevity parameters (in days) of mouse strains used in study.

	n	Mean (d ± SEM)	Median (d)	90% (d)
Control	35	835 (±22)	857	974
TgCyto MsrA	31	789 (±36)	789	1018
TgMito MsrA	23	865 (±28)	886	1008

We tested the median survivorship of each transgenic line against that of the control mice and found no significant effect for either TgCyto MsrA (p=0.08) or TgMito MsrA (p=0.43). As can be seen from Fig. 1, the TgCyto MsrA and TgMito MsrA curves appeared to suggest slightly greater maximum (90%) survivorship than that of control mice. Using the Fisher's Exact Test method to determine effect on maximum lifespan, we found that neither TgCyto MsrA (p=0.09) or TgMito MsrA (p=0.08) differed from control mice [21]. Maximum lifespan as determined by Boschloo's Exact test, also described by Wang and Allison, showed a significant increase in maximum lifespan of TgCyto MsrA mice in relation to control mice (p=0.018), but no difference between TgMito MsrA and control mice (0.09). These data then highlight both the importance of Gompertz analysis of lifespan as well as potential deficiencies that may occur when only a single method of longevity analysis is used.

Previous studies have suggested that transgene expression driven by certain promoter elements can decline with age in mouse models [22]. Here, we show that young TgCyto MsrA and TgMito MsrA mice express high levels of MsrA in kidney (relatively high in endogenous MsrA) and skeletal muscle (relatively low in endogenous MsrA) and that these are largely maintained with age (Fig. 2). Similar results were found in other tissues including liver and brain (data not shown). As previously reported, the level of overall MsrA expression is higher in TgMito MsrA mice than in TgCyto MsrA mice [17,19]. Increasing MsrA expression in either transgenic model does not significantly alter MsrB expression suggesting no compensatory changes in regulation of this Msr isoform. However, MsrB levels significantly decline with age to the approximately the same degree in all lines of mice.

As measures of healthspan, we addressed whether increased MsrA expression could improve the maintenance of metabolic homeostasis in aging mice including body composition and glucose metabolism. In these studies, young females were between 6 and 9 months and old females between 24 and 30 months of age. Old mice did not differ in body weight compared to young mice. Among age-matched mice we found no effect of genotype on body weight (Fig. 3). Despite the lack of effect on overall weight, we did find that aging promoted a shift in body composition with age; fat mass was significantly reduced and fat-free mass was significantly increased in old mice relative to that found in young mice (Fig. 3). Again, genotype had no significant effect on either of these parameters; that is, body composition of TgCyto MsrA and TgMito MsrA mice did not differ from age-matched control mice.

We recently reported that TgCyto MsrA and TgMito MsrA mice do not differ from control mice in glucose tolerance or insulin sensitivity when measured in young mice maintained on normal rodent chow [19]. However, we also showed that TgMito MsrA mice showed preserved insulin sensitivity when they were maintained on a high fat/high sugar diet. We interpret these findings as evidence that mitochondrial MsrA overexpression does not grossly affect glucose metabolism, but rather protects against the dysregulation of glucose metabolism associated with metabolic stress. We show here that old TgMito MsrA mice have increased sensitivity to insulin as measured by insulin tolerance test (Fig. 4). In particular, at the 90 and 120 min points of this test, TgMito MsrA mice show hypoglycemia due to injection of insulin whereas blood glucose levels begin to return to normal in control and TgCyto MsrA mice. This outcome in TgMito MsrA mice likely suggests increased relative hepatic insulin sensitivity in that there is a sustained insulin-mediated suppression of hepatic glucose production. In line with this, we found no effect of MsrA expression on glucose tolerance, the outcome of which is driven largely by insulin release and insulin-mediated glucose uptake in muscle. Fasting concentrations of insulin in the plasma were not markedly different between control and TgMito MsrA mice (control =0.59 ±

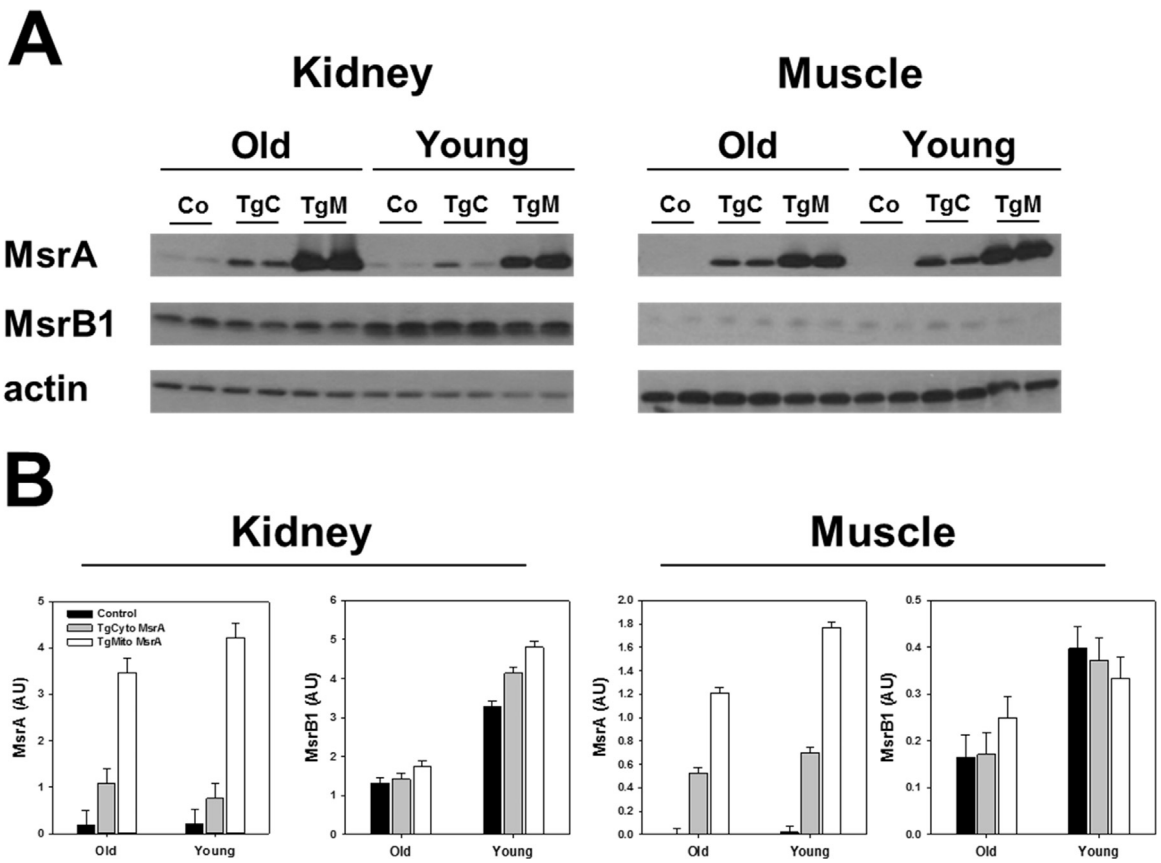

Fig. 2. MsrA and MsrB1 expression changes with age and transgenic expression. A. Representative immunoblot of kidney and muscle of young (6–9 months) and old (24–30 months) control (Co), TgCyto MsrA (TgC) and TgMito MsrA (TgM) mice. B. Quantification of proteins measure in A. Bars indicated mean protein level for indicated group; error bars indicated standard error.

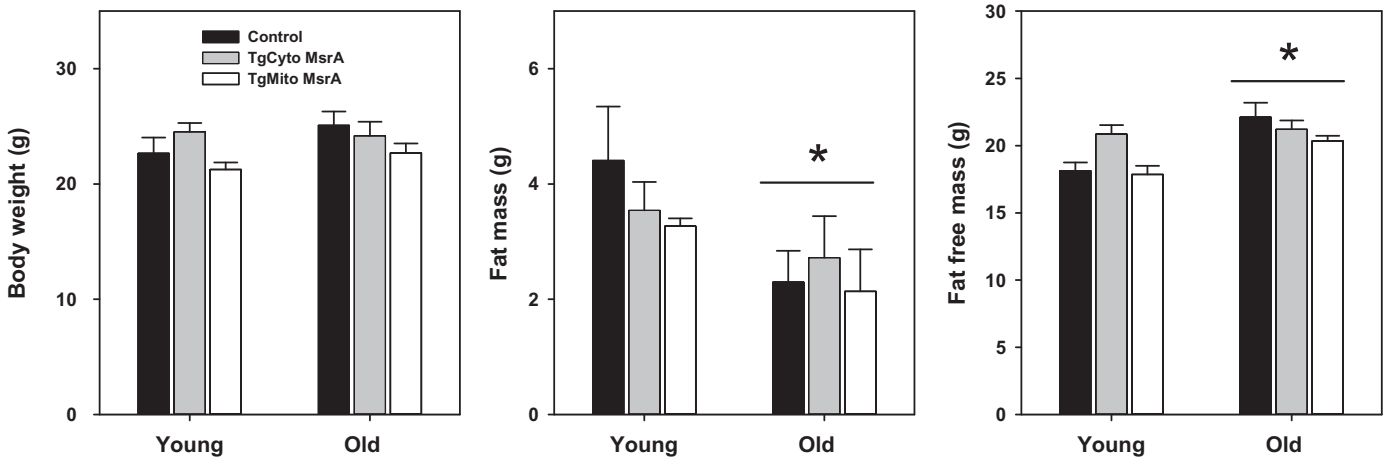

Fig. 3. Age, but not transgenic MsrA expression, alters body composition. Body weight, fat mass and fat-free mass from young (6–9 months) and old (24–30 months) control, TgCyto MsrA and TgMito MsrA mice. Bars indicate mean values (± standard error) for indicated measurements. Asterisks indicate significant effect of age.

0.17 ng/mL, TgMito MsrA=0.34 ± 0.08 ng/mL). These data suggest that increased mitochondria-targeted MsrA protects against age-related declines in insulin signaling, perhaps most importantly those that regulate hepatic glucose metabolism.

3. Discussion

We show here that overexpression of the protein repair enzyme MsrA in the cytosol, but not the mitochondria, can slow the rate of aging in the mouse. However, our data also show no significant effect on maximum or median lifespan in mice that overexpress MsrA in either the cytosol or mitochondria. These data are in contrast to the studies in *Drosophila* showing that the increased expression of either endogenous or bovine MsrA does significantly extend lifespan [12,14]. Our data is important because it indicates that the effect of MsrA overexpression on the lifespan on Drosophila does not translate

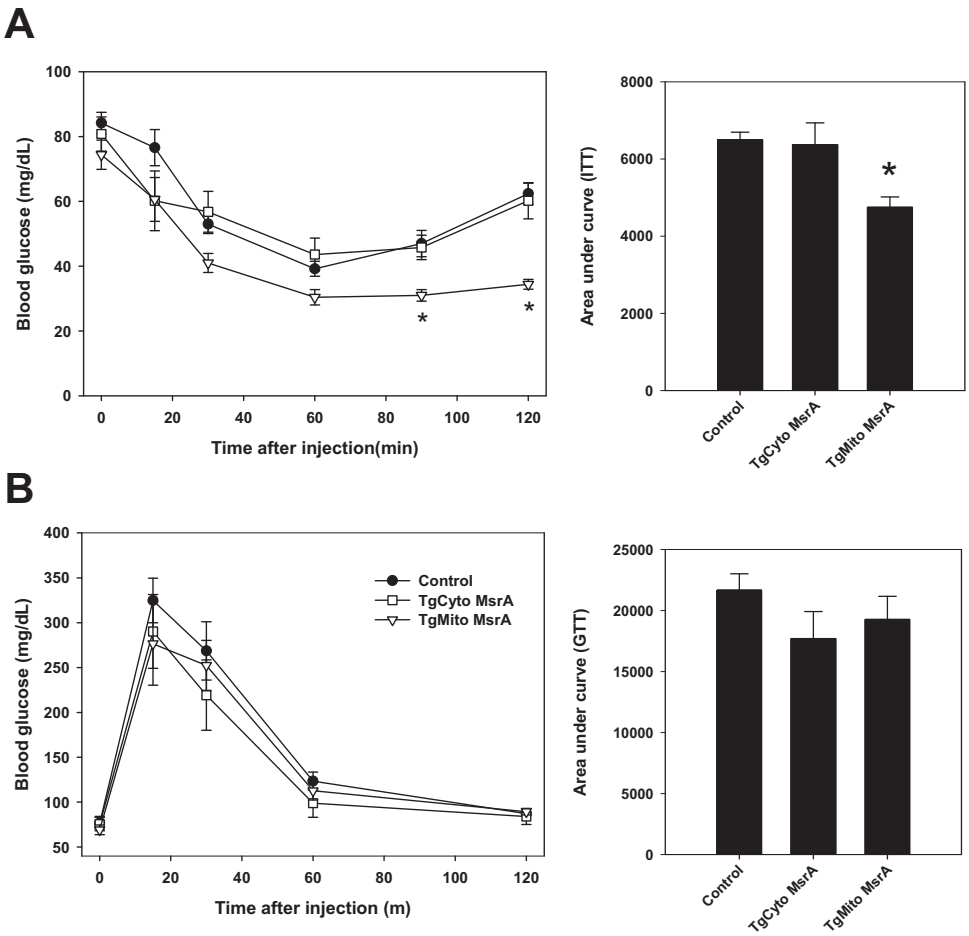

Fig. 4. Mitochondrial-, but not cytosolic-, targeted MsrA overexpression improves insulin sensitivity in old mice. A. Insulin tolerance tests and calculated areas under curves. B. Glucose tolerance tests and calculated areas under curve. For both, each symbol represents mean value (± standard error) for Control (closed circle), TgCyto MsrA (open square) and TgMito MsrA (open triangle) female mice for indicated tests. Bars indicate mean values (± standard error) for indicated measurements. Asterisk indicates significant effect of genotype.

directly to mammals. It is also important to note that we also did not see any effect of knocking out MsrA on the lifespan of mice [7]. A possible explanation for the inability of overexpression of MsrA to extend the mouse maximal lifespan is the difference in causes of death in flies and mice. The most likely primary cause of death in mice is cancer, but the causes of mortality in *Drosophila* are uncertain [23]. This might suggest that the primary cause of death in C57BL/6 mice (*i.e.* mostly lymphoma) may be largely unaffected by MsrA. However, MsrA has been shown to impact the progression of several diseases known to be accelerated by the aging process, including metabolic dysfunction, neurological diseases, breast cancer and retinal defects in mammals [19,24–27]. Moreover, we showed that increasing levels of MsrA in mice is protective against cardiac ischemia/reperfusion and metabolic dysfunction caused by obesity and/or aging [18,19]. One possibility is that oxidative stress plays a greater role in the modulation of these age-related physiological functions and does not largely drive death and/or longevity in mice. In the future, it will be necessary to delineate the roles that MsrA plays among multiple factors that affect healthy aging if not lifespan in mice.

Our data suggest physiological benefits with regard to healthy aging in mammals that are associated with increasing levels of MsrA. The decreased rate of aging in TgCyto MsrA mice as measured by Gompertz analysis and increased insulin sensitivity in aged TgMito MsrA mice are consistent with the interpretation of improved healthspan. In particular, using Gompertz analysis of longevity allowed us to determine a significant difference in the rate of aging despite no clear effect on overall survival of TgCyto MsrA mice when measured by Log Rank. These basic conclusions are summarized in Fig. 5. While we are unable to do so with the models used here, it would be of interest to test whether global overexpression of MsrA (*i.e.* in both cytosol and mitochondria) might extend both benefits on aging mice.

The aging process is associated with significant alterations in metabolic function in mammals, including dysfunctional glucose homeostasis, development of insulin resistance and alterations in energy utilization. In humans, the prevalence of diabetes is significantly higher in aged populations, even after accounting for changes in body mass, and is one of the most common co-morbidities in the elderly [28]. Thus, preventing metabolic dysfunction is likely to significantly improve healthy aging. Here and elsewhere, we have now defined a role for MsrA in regulating insulin sensitivity in response to both high fat diet and aging [19,24]. As recently reported, MsrA preferentially repairs methionine sulfoxide in unfolded proteins [29], and we might predict that the presence of mitochondrial MsrA plays an important role in the quality control during mitochondrial protein import. Following this idea, loss of MsrA in both the cytosol and mitochondria is associated with mitochondrial dysfunction [27]. Determination of the mechanisms by which MsrA affects mitochondrial function is a goal for future studies.

4. Methods

4.1. Animals

Mice with MsrA overexpression targeted to the cytosol (TgCyto MsrA) or mitochondria (TgMito MsrA) were bred in the animal facilities of the Audie L. Murphy Veterans Administration Hospital of the South Texas Veteran's Health Care System. Individual transgenic lines were maintained with hemizygous (*i.e.*, Tg/wt x wt/wt) breeding plan to generate both transgenic and wild-type control littermates. All strains of mice are maintained in the C57BL/6J genetic background. Mice were genotyped for presence of transgene, and overexpression was confirmed by immunoblot. For longevity studies, female mice were used and all cages of mice were maintained at a density of 4 mice per cage beginning at approximately 3 months of age. For littermate cages originally < 4, females of the same approximate age from different cages were supplemented to achieve a final number of 4 per cage. Mice were housed under barrier conditions using micro-isolator cages and were fed *ad libitum* a standard NIH-31 chow diet (Harlan Teklad, Madison, WI). Mice were maintained on a 12-h dark/light cycle in the animal vivarium. The mice in the survival groups were monitored daily and maintained until natural death. A separate cohort of mice were maintained under similar conditions to generate old animals for metabolic/healthspan assessments. All procedures involving mice were approved by the Subcommittee for Animal Studies at the Audie L. Murphy Veterans Administration Hospital at San Antonio and the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio.

4.2. Metabolic assessment

Glucose and insulin tolerance tests were performed after overnight fast (18:00–09:00 the following day) and then injection of either glucose (1.5 g kg^{-1}) or insulin (0.75 U kg^{-1}) intraperitoneally as previously described [19,24]. A handheld OneTouch Ultra glucometer (LifeScan, Inc., Milpitas, CA) was used to measure whole blood glucose at each indicated time point. For all other metabolic assessments, animals were transferred to the San Antonio Nathan Shock Center Healthspan and Functional Assessment core facility. Body composition was assessed in non-anesthetized mice by Quantitative Magnetic Resonance imaging (QMRi) using an EchoMRI 3-in-1 composition analyzer (Echo Medical Systems, Houston TX). Insulin concentrations were determined in plasma from fasting animals using kit from Crystal

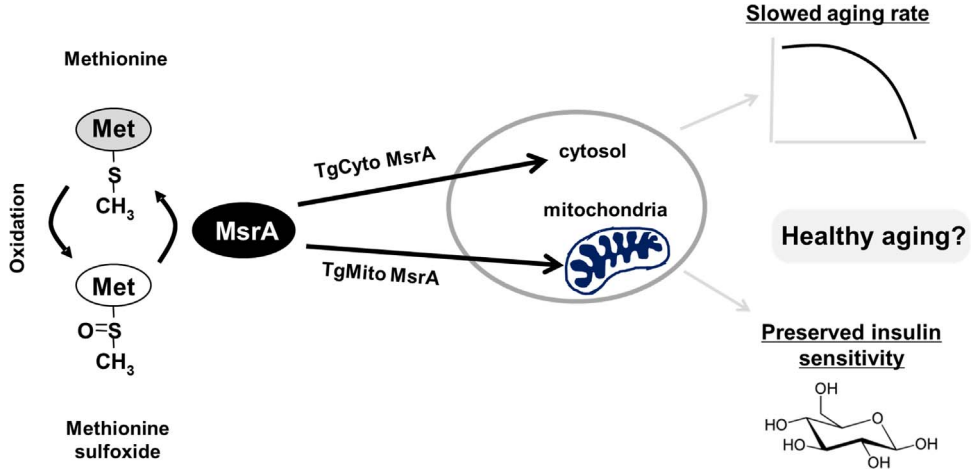

Fig. 5. Scheme of the major conclusions from this study.

Chem (Downers Grove, IL).

4.3. Immunoblotting

Tissue samples were collected from mice euthanized by CO_2 inhalation followed by cervical dislocation. All samples were frozen in liquid N_2 and stored at −80 °C until use. Protein extracts were prepared in RIPA buffer with added phosphatase and protease inhibitors (Thermo Scientific, Rockford, IL, USA). Standard protocols were used for separation of proteins by SDS-PAGE and transfer to PVDF membrane. After incubation with primary antibodies to MsrA and MsrB1 (Abcam, Cambridge MA) or actin (Sigma, St. Louis MO), alkaline phosphatase-conjugated secondary antibodies and ECL reagent were used to detect bands which were quantified using ImageJ software.

4.4. Statistical analysis

Longevity curves were determined using the Kaplan-Meyer estimator and analyzed for difference using log-rank and Gehan-Breslow tests. Maximum lifespan was analyzed using the Wang-Allison test [21] which uses a Boschloo's Exact test to test differences between groups in their survival of maximum 10% of subjects with the longest lifespan. The Gompertz mortality analysis of lifespan was conducted in order to model the hazard rate variation in time. The Gompertz distribution, with shape parameter *a* and scale parameter *b,* has instantaneous age-specific mortality (hazard rate) given by $h(t)=a\ exp(bt)$, and it is known to provides a remarkably close fit to adult mortality. The Gompertz parameters a and b are the initial rate of mortality and the rate of increase in the hazard for mortality (*i.e.* the mortality rate), respectively. Log hazard function dependence on time is linear, with intercept log(a) and slope b, and can be easily visualized on a 2d Gompertz plot. Kaplan–Meier estimation, Gompertz parametric modeling, log-rank testing for differences between survival curves, and Boschloo testing for difference in extreme survival were performed with specific functions implemented in the R packages survival, eha and Exact. Quantile regression, as described in Koenker [30] and implemented in the R package quantreg, was used to estimate and compare mean, median, and extreme quartile survival times for each group. For glucose and insulin tolerance tests, areas under curves were calculated using the trapezoid method. Differences among groups were determined by ANOVA. For all other tests, two way ANOVA were used to test for differences in age and genotype. Post-hoc analysis was performed using the method of Holm-Sidak.

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