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Mitochondrial peptidase IMMP2L mutation causes early onset of age-associated disorders and impairs adult stem cell self-renewal

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Summary

Mitochondrial reactive oxygen species (ROS) are proposed to play a central role in aging and age-associated disorders, although direct *in vivo* evidence is lacking. We recently generated a mouse mutant with mutated *inner mitochondrial membrane peptidase 2-like* (*Immp2l*) gene, which impairs the signal peptide sequence processing of mitochondrial proteins cytochrome c1 and glycerol phosphate dehydrogenase 2. The mitochondria from mutant mice generate elevated levels of superoxide ion and cause impaired fertility in both sexes. Here, we design experiments to examine the effects of excessive mitochondrial ROS generation on health span. We show that *Immp2l* mutation increases oxidative stress in multiple organs such as the brain and the kidney, although expression of superoxide dismutases in these tissues of the mutants is also increased. The mutants show multiple aging-associated phenotypes, including wasting, sarcopenia, loss of subcutaneous fat, kyphosis, and ataxia, with female mutants showing earlier onset and more severe age-associated disorders than male mutants. The loss of body weight and fat was unrelated to food intake. Adipose-derived stromal cells (ADSC) from mutant mice showed impaired proliferation capability, formed significantly less and smaller colonies in colony formation assays, although they retained adipogenic differentiation capability *in vitro*. This functional impairment was accompanied by increased levels of oxidative stress. Our data showed that mitochondrial ROS is the driving force of accelerated aging and suggested that ROS damage to adult stem cells could be one of the mechanisms for age-associated disorders.

Key words: adult stem cell; aging; *Immp2l* mutation; mitochondria; reactive oxygen species (ROS); superoxide.

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Introduction

Mitochondria are an important source of endogenous reactive oxygen species (ROS) (Kowaltowski *et al.*, 2009), which can damage macromolecules such as DNA, proteins, and lipids. ROS originating in the mitochondria were proposed by Harman as a major cause of aging (Harman, 1956, 1972; Sedensky & Morgan, 2006). Numerous studies in yeast and *Caenorhabditis elegans* supported the free radical theory of aging. For example, knocking out Sod1 in yeast shortens lifespan (Wawryn *et al.*, 1999) and treating *C. elegans* with small synthetic superoxide dismutase/catalase mimetics increases lifespan (Melov *et al.*, 2000). However, some studies in Drosophila (Seto *et al.*, 1990; Bayne *et al.*, 2005) as well as in mice (Huang *et al.*, 2000; Lapointe *et al.*, 2009; Perez *et al.*, 2009) do not support this theory.

Mitochondrial ROS have been implicated in age-related disorders such as atherosclerosis and cardiovascular disease (Madamanchi & Runge, 2007), insulin resistance (Wiederkehr & Wollheim, 2006), and age-related neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington disease, and amyotrophic lateral sclerosis (ALS) (Chen *et al.*, 2006; Lin & Beal, 2006; Martin, 2006; Zhou *et al.*, 2008a). Despite the evidence for mitochondrial ROS involvement in these disorders, it still remains to be established whether mitochondrial ROS are a causal factor or arise secondarily to the disorders (Swerdlow, 2007).

Superoxide dismutase 1 (SOD1) and SOD2 both contribute to the detoxification of mitochondrial-generated superoxide. SOD1 localizes to the cytosol and the mitochondrial intermembrane space (IMS) and is involved in scavenging superoxide in these areas (Okado-Matsumoto & Fridovich, 2001; Han *et al.*, 2003). SOD2, on the other hand, localizes to the matrix of the mitochondria and is involved in scavenging superoxide in that location. The role of these major antioxidant enzymes in aging is currently controversial. Some studies have reported a decrease in the expression or activities of these enzymes during aging (Rao *et al.*, 1990; Sandhu & Kaur, 2002; Chehab *et al.*, 2008). Others have found that expression levels were either unchanged or even slightly increased during aging (Liu & Mori, 1993; Sohal *et al.*, 1994; Hussain *et al.*, 1995; Sasaki *et al.*, 2008).

Recently, we generated an *Immp2l*$^{Tg(Tyr)979Ove}$/*Immp2l*$^{Tg(Tyr)979Ove}$ mouse model with a mutation in the *inner mitochondrial membrane peptidase 2-like* (*Immp2l*) gene (Lu *et al.*, 2008). IMMP2L is a subunit of mitochondrial inner membrane peptidase, a heterodimer complex exclusively located in the mitochondrial inner membrane that cleaves the intermembrane space-sorting signals from the precursor or intermediate polypeptides after

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they reach the inner membrane or the intermembrane space (Behrens *et al.*, 1991; Nunnari *et al.*, 1993). Mammalian IMMP2L has two known substrates, cytochrome c1 and mitochondrial glycerol phosphate dehydrogenase 2, and *Immp2l* mutation impairs the signal peptide sequence processing of both proteins (Lu *et al.*, 2008). Consistent with the finding that both proteins are involved in superoxide generation (Brand, 2010), the mitochondria from mutant mice generate elevated levels of superoxide ion, but show no obvious deficiencies in ATP generation and membrane potential maintenance. Although mutant males present with impaired spermatogenesis only after the age of 7 months, they are infertile throughout their lives because of erectile dysfunction (Lu *et al.*, 2008). Female mutants are also infertile because of impaired oogenesis. In addition, aged mutants show impaired bladder function (Soler *et al.*, 2010). All these phenotypes observed in mutant mice can be explained by excessive generation of mitochondrial superoxide ion, which can inactivate nitric oxide, an messenger molecule important for penile erection, oogenesis, and bladder function, and form other forms of ROS, which can damage DNA, lipids, and protein (Lu *et al.*, 2008).

The finding of elevated superoxide generation in *Immp2l* mutant mice prompted us to hypothesize that *Immp2l* mutant mice might develop a number of health issues and/or have a compromised health span if mitochondrial ROS are involved in aging. Here, we tested this hypothesis by examining whether the health of mutant mice is compromised. Control and mutant mice were housed under optimal conditions up to the age of 30 months, and the presentation of age-associated disorders was recorded. We found that *Immp2l* mutant mice manifest multiple aging-associated phenotypes that were not observed in age-matched normal control mice, including wasting, loss of subcutaneous fat, sarcopenia, kyphosis, and ataxia, beginning at 16 months of age. The loss of subcutaneous fat is the result of impaired self-renewal of adipose progenitor/stem cells. Our data demonstrate that excessive mitochondrial ROS compromise the health of the mutant animals and suggest that the effects of excess ROS on murine health are at least partially the result of impaired self-renewal of adult stem cells.

Results

Multiple tissues of mutant mice show elevated oxidative stress despite increased expression of ROS scavenger enzymes

Previously, we observed a higher than normal rate of superoxide generation by isolated mitochondria from mutant tissues. In these studies, we proposed that the resultant oxidative damage underlies the observed infertility, bladder dysfunction, and age-dependent spermatogenic disruption (Lu *et al.*, 2008; Soler *et al.*, 2010). To substantiate our hypothesis, we examined the presence of oxidative stress in multiple tissues of aged mutants by examining the expression of 4-hydroxynonenal (HNE), a product of protein oxidative modification (Yoritaka *et al.*, 1996). As expected, we detected elevated HNE-protein adduct expression in the brain and the kidney of the mutants (Fig. 1A). Consistent with the ubiquitous expression of mutated *Immp2l*, higher HNE-protein adduct was also found in the testicular tissue of mutant mice (our unpublished data). These data demonstrate that there are elevated levels of ROS and oxidative stress in multiple mutant tissues.

The role of major antioxidant enzymes in aging is currently controversial. We examined SOD1 and SOD2 expression in mutant and control animals and found that expression of both enzymes is significantly elevated in the kidney and brain of mutant mice (Fig. 1B,C). Similar observations were also made in the testis (our unpublished data), suggesting the effects of *Immp2l* mutation are global. Our data demonstrate that with elevated mitochondrial ROS generation, expression of major ROS scavenger enzymes is also elevated, although the overall cellular oxidative stress is increased. Our data support the view that increased oxidative stress during aging is mainly caused by enhanced ROS production rather than decreased ROS scavenger activities (Sasaki *et al.*, 2008).

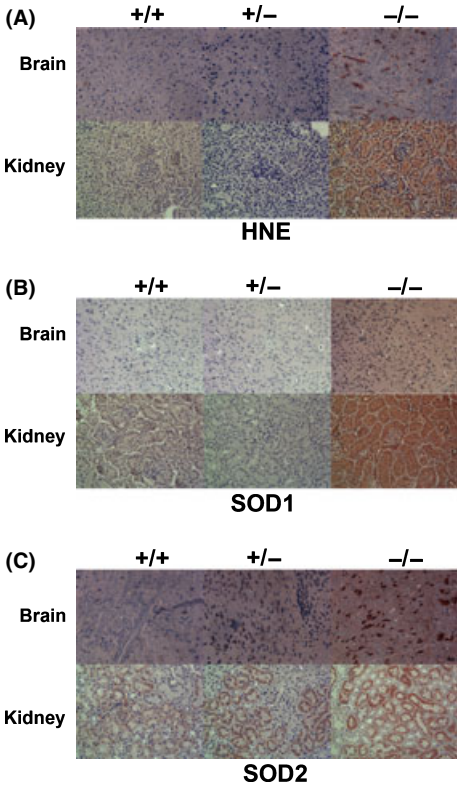

Fig. 1 Elevated expression of 4-hydroxynonenal-protein adducts and SOD in mutant tissues. (A) HNE-protein adducts expression was significantly elevated in the brain and kidney of mutant mice. (B) SOD1 expression was increased in the brain and kidney of mutant mice. (C) Increased SOD2 expression in the brain and kidney of mutant mice. Brain and kidney tissues were from females of 16 months. Positive signals were visualized by 3-amino-9-ethylcarbazole, which stained reddish-brown. Nuclei were counterstained with hematoxylin. +/+, +/–, and –/– indicate normal control, heterozygous, and homozygous mutant mice. Shown were typical staining results for tissues from at least three animals of each genotype.

Early onset of aging-associated disorders in mutant mice

The presence of elevated ROS stress in multiple tissues of mutant mice prompted us to propose that early onset of aging-associated disorders should be observed if ROS play a major role in aging. We housed the mutants and their littermates under optimal conditions for up to 30 months. Mutant mice are apparently normal in terms of development, locomotion, grooming, interaction between cage mates, and aggressive reaction to intruders (males) before the age of 16 months. However, multiple signs of accelerated aging are observed in mutant mice after the age of 16 months. Although both sexes are affected by *Immp2l* mutation, female mutants show greatly exaggerated phenotypes. The following description will focus on observations made on mutant females; differences between sexes will be specified wherever they are observed.

Slight kyphosis can be observed as early as 16 months after birth in the mutant females, which becomes more pronounced at the age of 24 months (Fig. 2A). Starting at the same time, mutant females show ataxia, which become more severe over time (see supplementary Video S1). Body weights of mutant females show no significant differences from control mice at 2–3 months, while they are significantly smaller than those of control mice thereafter (Fig. 2B). The control mice gain significant weight up to the age of 20–24 months, while the mutants gain little weight after the age of 6–7 months and started to lose weight after the age of 20–22 months. Food intake of 6- to 7-month-old mutants is not reduced if expressed as percentage of body weight (Fig. 2C). Thus, it is unlikely that the mutants fail to gain weight because of reduced energy intake or malnutrition.

Measurement of absolute weights of the soleus and gastrocnemius muscles from the hind legs of mutant females indicates that there is no difference in the size of these muscles before the age of 10 months (data not shown), but by the age of 20–24 months (Fig. 2D), the weight of both muscles is significantly lower in the mutant mice, indicating early onset of sarcopenia. Here, absolute muscle weight rather than muscle weight/body-weight ratio is used, because the body weight of aged mutant mice decreases greatly. Because the absolute weights of the soleus and gastrocnemius muscles from the control and mutant mice show no difference before the age of 10 months, it is highly likely that that the decrease in muscle mass in aged female mutants is the result of sarcopenia. Histologic examination of the skeletal muscle from mutant mice reveals no abnormalities in terms of myocyte size, nuclei localization, and infiltration of lymphocytes (Fig. 2E). However, more work is needed to determine whether the muscle tissues from aged mutants have other defects. Consistent with obvious sarcopenia, wire hang testing, which examines the ability of the mice to grasp a metal mesh and the strength of their grip, reveals that mutant females have significantly shorter holding times when they hang from the inverted wire mesh (Fig. 2F).

The inability of the mutant mice to gain weight after 6–7 months is due partially to their inability to accumulate as much fat as the controls. The white fat pads in mutant females are a normal size at 2–3 months of age (data not shown). However, by 10–12 months of age, the white fat pads in the mutant females are just 1/3 the size of those in age-matched control females (Fig. 3A). In some of the aged mutant females, gonadal fat and visceral fat pads are barely visible. Mutant females also have subcutaneous fat before the age of 20 months (Fig. 3D), but most female mutants completely lose their subcutaneous fat after 20 months of age. In contrast, control mice retain their subcutaneous fat at the same age (Fig. 3E). Adipose tissue histology reveals that at the ages of 10 months and 24 months, the remaining adipocytes are fully differentiated with normal size (Fig. 3B,C), indicating that hypoplasia (reduced cell number) likely explains the reduced adipose mass. This also suggests that beta-oxidation is unlikely to be involved because increased beta-oxidation would predict a reduction in adipocyte size (Jeong & Yoon, 2009).

Male mutant mice are also affected by this mutation, but they present with age-associated disorders at later times and with reduced severity compared to the female mutants. For example, males do not show kyphosis and ataxia until after the age of 24 months. Their body weights only show significant differences from control mice after the age of 6–7 months, and the reduction in body weight occurs to a lesser degree in the males (Fig. 2G). Both of the male mutants retained subcutaneous fat even at the age of 24 months, and their fat depots were only reduced to half the size of those seen in control mice even at this age. The most significant difference between male and female mutants lies in the skeletal muscle, in that sarcopenia is not observed in male mutants even at the age of 24 months. Because most of the male animals were used to examine the male reproductive system before the age of 24 months in another study, observation of age-associated disorders in male mutants beyond this age was not attempted because of the limited number of male animals available for study.

Interestingly, we did not notice an increase in tumor development in mutant mice, despite the fact that increases in ROS and oxidative stress have been proposed to increase the risk of tumorigenesis. However, a more comprehensive survey of tumorigenesis in the mutant mice is needed to fully understand the role of ROS in tumorigenesis. Our observation that there was no increase in tumor occurrence in *Immp2l* mutant mice is not surprising. It has been reported that glutathione peroxidase 4 deficiency causes elevated ROS stress, but still decreases the overall risk of tumorigenesis because the mutant cells are more sensitive to apoptosis (Ran *et al.*, 2007). Interestingly, we also observed increased germ cell apoptosis in mutant males (Lu *et al.*, 2008).

The role of ROS in the progression of type II diabetes is under active investigation. Our preliminary data indicate that *Immp2l* mutant mice have normal glucose maintenance at the age of 10 months (our unpublished data). More work is needed to determine glucose levels and insulin sensitivity in mutant animals at later ages, and these studies are currently underway in this laboratory.

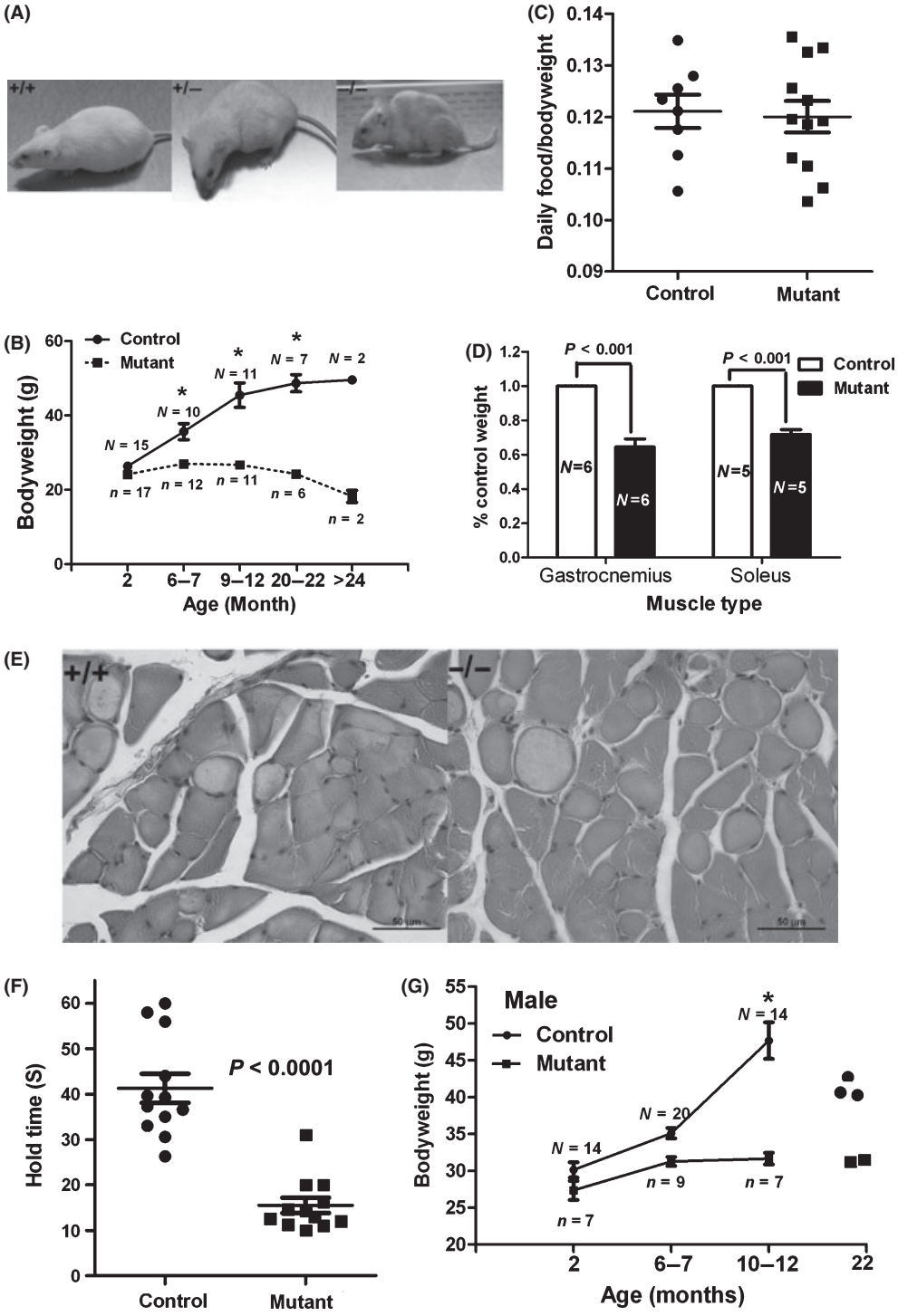

Fig. 2 Aging-associated phenotypes in mutant mice. (A) Kyphosis of 24-month-old female mutant mice. Note the difference of coat color because of different expression of transgenic tyrosinase in heterozygous (+/−) and mutant (−/−) mice. (B) Reduced body weight of aged mutant females. Number of animals of each time point is listed. Control mice include +/+ and +/− genotypes. * indicates significant difference by ANOVA. Animals beyond 24 months were excluded from ANOVA analysis because of limited sample number. (C) No difference in food consumption between 6 and 7 months female control and mutant mice. (D) Reduced skeletal muscle mass of 20–22 month mutant females. Numbers of samples are listed in the columns. As each time one mutant and one age-matched control were sacrificed at the same time, muscle mass from the mutant was expressed as fraction of that of the control. Significant difference was found between control and mutants by ANOVA. (E) H&E staining of soleus muscle from 21-month females. (F) Wire hang assay showed reduced grip strength of mutant mice at 21 months. (G) Body weight of mutant males. Significant difference can only be observed at 10–12 months (ANOVA). Available bodyweight data beyond 10–12 months are also presented in the figure but these data are excluded from ANOVA analysis because of the limited number of samples. * indicates significant difference by ANOVA. Means ± SEM are represented in B, C, D, E and G.

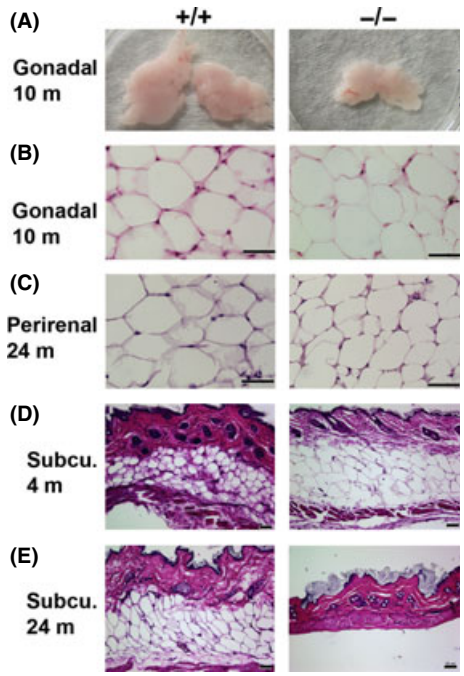

Fig. 3 Loss of adipose tissue in aged mutant females. (A) Smaller gonadal adipose tissue of 10-month mutant females. (B) H&E staining of gonadal fat tissue of 10-month females. No difference in size of adipocytes is noted between control and mutants. (C) H&E staining of perirenal fat tissue of 24-month females. No difference in size of adipocytes is noted between control and mutants. (D, E) H&E staining of skin cross-section showing the loss of subcutaneous fat in 24-months but not 4-months mutant females. Scale bars: 50 μ.

Impaired self-renewal of adipose progenitor cells in mutant mice

Because malnutrition did not explain the fat tissue loss in aged mutant females, and because it appears that a reduction in adipocyte number underlies the reduced fat mass in these animals, we examined whether the adipose progenitor cells in the mutant mice were defective. We isolated adipose-derived stromal cells (ADSC) from gonadal fat of mutant and control females aged 24 months and differentiated these cells toward adipocytes *in vitro*. ADSC from control mice gave rise to clusters of adipocytes which could be stained red by Oil Red O, indicating that they contain fat. However, these stained clusters were rarely observed in cultures of mutant mice (Fig. 4A). Some single Oil Red O-stained cells could be observed in the mutant cultures, which indicated that the induction medium for the mutant cells is functional. These data show that ADSC cells from mutant females have defects.

We postulated that one such defect could be the reduced number and impaired proliferation capability of White Adipose Tissue (WAT) progenitor cells from mutant mice. To test our hypothesis, we isolated ADSC cells from gonadal fat of female mice and performed colony formation assays. At 5–8 days after plating, numerous colonies of > 50 cells could be observed in control cultures, whether the cells were isolated from control mice of 10 months or 24 months of age. However, the number and the size of the colonies formed were significantly reduced in cultures from mutant mice, and this was especially evident in cultures derived from mutant mice at 24 months (Fig. 4B). In *in vitro* proliferation assays, ADSC cells from mutant mice had longer doubling times and ceased to proliferate earlier than ADSC cells from normal control mice. This was observed in ADSC cells from 24-month-old mutants (Fig. 4C) and 10-month-old mutants (data not shown), although it was less pronounced in cells from 10-month-old mutants.

We performed senescence-associated (SA) β-galactosidase staining to compare cell senescence in cultured ADSC cells from control and mutant gonadal fat. In some cases, we saw no difference in the percentage of SA-gal-positive cells between the two genotypes, while in other cases we found even more SA-gal-positive cells in control cultures (data not shown). The data suggest that cell senescence during *in vitro* culture cannot explain the observed proliferation difference. Because SA-galactosidase is expressed in senescent cells, but not in terminally differentiated cells (Dimri *et al.*, 1995), fewer SA-galactosidase-positive cells in cultures from mutant mice may reflect the scarcity of proliferating progenitor cells in the mutant cultures.

To examine whether the defects in adult progenitor cell proliferation are unique to adipose tissue, we isolated bone marrow-derived stromal cells (BMSCs) from mutant and control mice and compared their proliferation capability *in vitro*. BMSCs from mutant mice also showed impaired proliferation capability, although this was not as pronounced as in adipose-derived cells (Fig. 4D). These data suggest that adult stem cells from various organs are affected by the mutation to varying degrees. This could also explain the observation of multiple aging-associated disorders in the mutant mice.

Elevated oxidative stress in ADSC cells from mutant mice

We propose that the observed defects in progenitor cells of mutant mice result from elevated oxidative stress caused by increased superoxide generation by mitochondria. If this is the case, oxidative stress would be increased in isolated ADSC cells from mutant mice. ADSC cells from 10-month-old mutant and control females were isolated and cultured *in vitro*. The level of HNE-protein adducts (an indication of lipid peroxidation) in these cells was compared by immunocytochemistry. As shown in Fig. 5A (top panel), ADSC cells from mutant mice showed significantly elevated levels of lipid peroxidation as judged by the elevated expression of HNE-protein adducts. In addition, cells from mutant mice had significantly decreased levels of glutathione (GSH) assayed by a fluorescent dye monochlorobimane (MCB) reacting with reduced GSH (Fig. 5B), indicating elevated oxidative stress in ADSC cells from mutant mice. Consistent with elevated mitochondrial superoxide generation in ADSC cells from mutant mice, their expression of SOD1 and SOD2 enzymes was adaptively increased (Fig. 5A, middle and bottom panels). Elevated lipid peroxidation was also observed in bone marrow-derived stromal cells (data not shown).

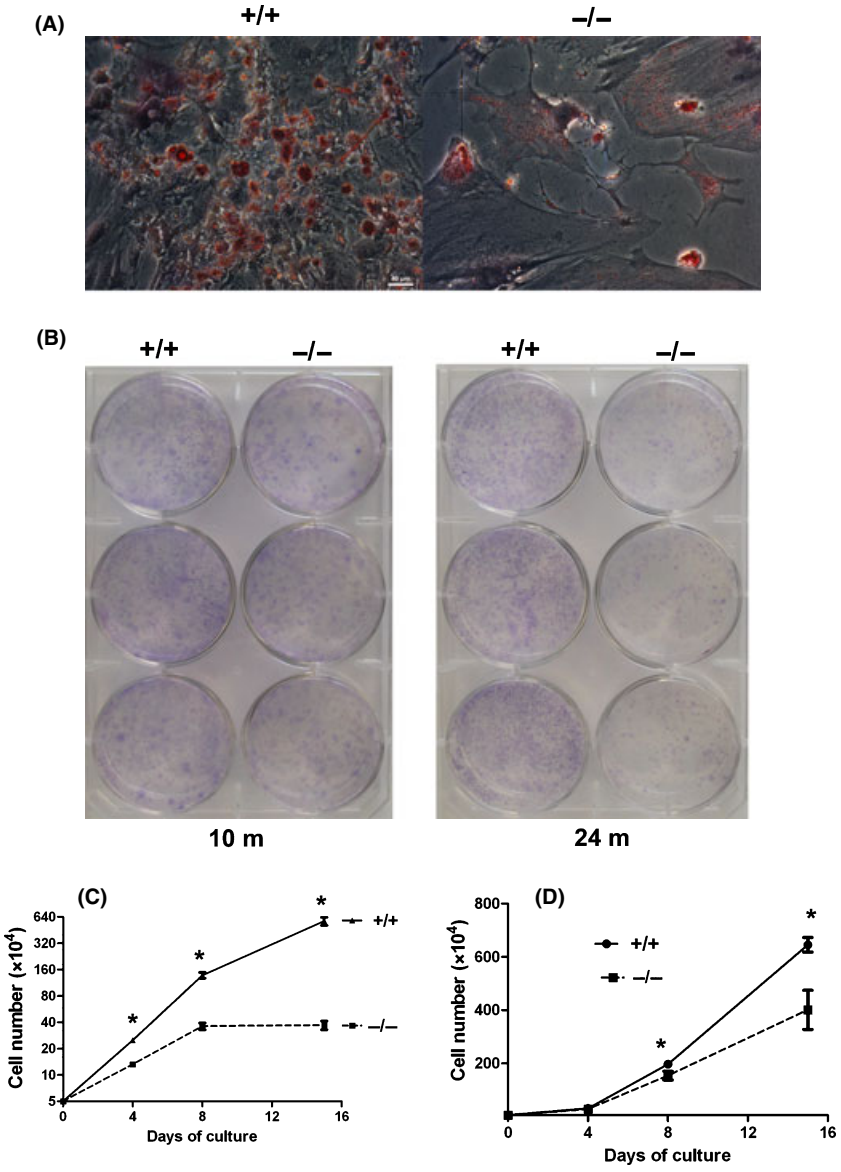

Fig. 4 Adult stem cell defects in mutant animals. (A) Adipogenesis was impaired in adipose-derived stromal cells (ADSCs) from mutant mice. Mature adipocytes were stained with Oil Red O. (B) ADSC cells from mutant mice formed fewer and smaller colonies in colony formation assay. Giemsa staining was performed to help visualize the cells. Differences were observed in ADSCs from gonadal adipose tissue of 10-month-old mice, but were more pronounced in ADSCs from gonadal adipose tissue of 24-month-old mice. (C) Proliferation assays for ADSC cells from control and mutant mice. (D) Proliferation assays for bone marrow-derived stromal cells from control and mutant mice. For (C) and (D), triplicate assays were performed for each experiment. The experiments were repeated at least three times and similar results were observed. Data from one experiment are shown; mean ± SEM.*, $p < 0.05$.

Discussion

The present study used a unique mouse model, where the primary defect is elevated mitochondrial superoxide generation, to examine the role of mitochondrial ROS in aging. We found that excessive mitochondrial superoxide generation caused by *Immp2l* mutation increases cellular oxidative stress and causes early onset of age-associated disorders and impaired self-renewal of adult stem cells. We believe that our work enhances our understanding of the roles of ROS in human aging in the following ways:

First, we show that the rate of ROS generation plays a more important role in aging than changes in ROS scavenger activities. It is still an open question as to whether increased oxidative stress during aging is mainly caused by enhanced ROS production or decreased ROS scavenger activities, or both (Rao *et al.*, 1990; Liu & Mori, 1993; Sohal *et al.*, 1994; Hussain *et al.*, 1995; Sandhu & Kaur, 2002; Chehab *et al.*, 2008; Sasaki *et al.*, 2008). We found that although major ROS scavenger enzyme expression is increased in our *Immp2l* mutant mice, cellular oxidative stress is still elevated, supporting the notion that increased oxidative stress during aging is mainly caused by enhanced ROS production rather than decreased ROS scavenger activities (Sasaki *et al.*, 2008).

Second, we demonstrated that mitochondrial free radicals are a major contributor to age-related disorders. The mitochondrial free radical theory of aging is currently being questioned owing to findings that are incompatible with the theory (Gruber *et al.*, 2008; Lapointe & Hekimi, 2010). One of the discrepancies is that genetic overexpression or inactivation of ROS scavenger enzymes does not change lifespan as predicted by the theory (Huang *et al.*, 2000; Lapointe *et al.*, 2009; Perez *et al.*, 2009). Our data demonstrate that *Immp2l* mutation and the resultant mitochondrial ROS overproduction causes early onset of ataxia,

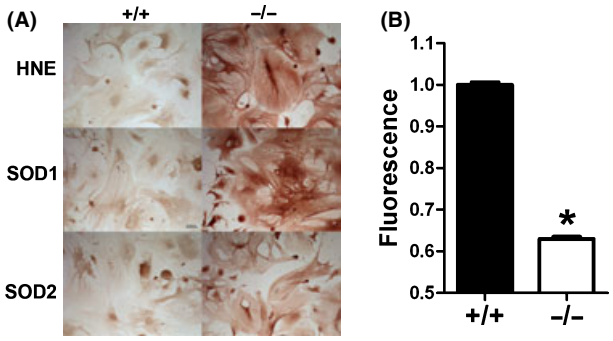

Fig. 5 Elevated oxidative stress in adipose-derived stromal cells from mutant mice. (A) Elevated expression of HNE-protein adducts and superoxide dismutase 1 and 2 in mutant adipose-derived stromal cells. (B) Lower glutathione (GSH) concentrations in mutant adipose-derived stromal cells. *, $p < 0.05$.

cachexia (fat loss and sarcopenia), weight loss, kyphosis, and loss of subcutaneous fat, all of which are observed in elderly humans and aging animals (Chumlea *et al.*, 1989; Haines *et al.*, 2001; Hughes *et al.*, 2004; Trifunovic *et al.*, 2004). Because the primary defect in the mutant mice is an elevated generation of mitochondrial superoxide and otherwise normal mitochondrial function (Lu *et al.*, 2008), our data support a major role for mitochondrial free radicals in affecting animal health span.

Third, our data suggest that mitochondrial ROS accelerate aging partially through impairment of the self-renewal of adult stem cells. Aging is accompanied by a steady decline in the number of adult stem cells (D'Ippolito *et al.*, 1999; Caplan, 2007; Tokalov *et al.*, 2007) and an impaired functionality of adult stem cells (Stenderup *et al.*, 2003; Karagiannides *et al.*, 2006; Stolzing *et al.*, 2008; Zhou *et al.*, 2008b). *In vitro* studies have shown that antioxidants accelerate growth and prolong the lifespan of adipose tissue-derived human mesenchymal stem cells (Lin *et al.*, 2005; Fehrer *et al.*, 2007) and that mitochondrial ROS are involved in senescence of adult stem cells (Heo *et al.*, 2009; Kasper *et al.*, 2009). However, *in vivo* evidence for a possible role of ROS in the aging of adult stem cells is missing. We found that hypoplasia accounts for the reduced fat mass in aged *Immp2l* mutant mice; furthermore, we observed impairment in the self-renewal of ADSC from mutant mice. Although ADSC are mixtures of adipose progenitor cells and differentiated cells at various stages, adipose progenitor cells (stem cells in the adipose tissue) contribute most to the proliferation and colony formation capability. The data suggest that inability of adipose progenitor cells to replace lost mature adipose cells as a result of defects caused by oxidative stress in progenitor cell self-renewal is one of the mechanisms underlying the hypoplasia of adipose tissue in aged mutants. However, the possible contribution of ROS-induced mature adipocyte apoptosis in reduced adipose mass in aged mutants cannot be excluded, although this was not examined in the present work.

Impaired stem cell self-renewal is not unique to ADSC in aged mutant mice. It was also observed in bone marrow-derived stromal cells. Adult stem cells exist in multiple systems, including skeletal muscle (satellite cells), brain (neural stem cells), and blood (hematopoietic stem cells). It remains to be seen whether mitochondrial ROS will have similar detrimental effects on these other types of adult stem cells. We observed defects in the nervous (ataxia) and skeletal muscle (sarcopenia) systems of aged mutant mice. In skeletal muscle from aged mutant mice, reduced myocyte number underlies the observed sarcopenia. It is likely that ROS exert detrimental effects to multiple types of adult stem cells and impair the regeneration of the aging tissues, which eventually accelerates aging. Thus, our data suggest that damaging adult stem cells could be one of the mechanisms through which elevated ROS accelerates aging. Experiments are currently underway to examine the effects of *Immp2l* mutation on adult stem cells of various tissue origins to test this hypothesis.

One of the strengths of the present work is that the *Immp2l* mutant model shows age-associated disorders in multiple systems and most of the disorders are observed after the age of 16 months. Thus, accelerated aging is most likely the result of ROS damage in multiple systems of the body rather than impairment in just one or two organs. The relatively long lifespan of *Immp2l* mutant mice compared with other aging mouse models, such as the mtDNA mutator mouse model, means that *Immp2l* mutant mice better resemble the aging process seen in humans.

Another strength of the present work is that the observations were made in a unique animal model with a primary defect in mitochondrial superoxide generation with otherwise normal mitochondria. Because of the unavailability of mouse models with specific enhanced mitochondrial ROS generation, current studies examining the involvement of ROS in aging use animal models with enhanced or diminished expression of major antioxidant enzymes such as SOD1 (Huang *et al.*, 2000; Elchuri *et al.*, 2004; Perez *et al.*, 2009), SOD2 (Li *et al.*, 1995; Zhang *et al.*, 2009), SOD3 (Sentman *et al.*, 2006), Gpxs (Neumann *et al.*, 2003; Yant *et al.*, 2003), Thioredoxin 1 (Matsui *et al.*, 1996), and Thioredoxin 2 (Nonn *et al.*, 2003). While these models are useful in addressing situations involving changes in ROS clearance rates, they are not suitable for situations that may involve enhanced ROS generation, the latter of which is more likely in aging and age-related disorders (Sasaki *et al.*, 2008). The roles of ROS scavenger enzymes would be best appreciated under situations of perturbed ROS generation. As in our mutant mice, SOD1 and SOD2 are up-regulated to counteract the elevated generation of mitochondrial superoxide, even though the end result is still increased cellular oxidative stress. To the best of our knowledge, the IMMP2L-deficient mouse model is the only currently available model with primary defect of elevated mitochondrial superoxide generation, yet obvious complicating other mitochondrial defects have not been noted so far.

One weakness of the present work is that the possible effect of elevated mitochondrial superoxide generation on lifespan is not addressed in this study. However, we did notice that some of our mutant mice lived up to 900 days before they were sacrificed because of severe ataxia. A well-designed longevity study is needed to determine whether elevated mitochondrial

superoxide generation will affect lifespan and if so, to what extent. Another weakness is that the possibility of antioxidants to rescue the compromised health span is not tested. For this purpose, the introduction of a mitochondria-targeted catalase transgene into *Immp2l* mutant mice will be informative. Furthermore, the signal pathways through which mitochondrial ROS damage adult stem cells and accelerate aging are not examined. Because excessive superoxide will generate elevated levels of peroxynitrite and we observed higher levels of protein modification by 4-hydroxynonenal in mutant mice, the possible involvement of protein tyrosine nitration and hydroxynonenal modification in this process need to be studied. These questions will be addressed in our future studies.

Striking differences are observed in female and male mutants with regard to the severity and onset of the age-associated disorders. Female mutants show greater bodyweight loss, earlier and more severe presentation of ataxia, and fat depot-loss than mutant males. In addition, female mutants show significant sarcopenia at the age of 20–24 months, when male mutants do not present a similar phenotype. The mechanism for the gender difference in sensitivity to mitochondrial ROS damage is unknown and warrants further study. Estrogen plays very important roles in adipose development at various stages of animal life (Cooke & Naaz, 2004); it has also been suggested to play a role in sarcopenia (Maltais *et al.*, 2009). It is known that estrogen levels decrease significantly in women and female animals during aging (Maffucci & Gore, 2006). In elderly men, bioavailability of estrogen also decreases but the level in aging men is significantly higher than in post-menopausal women (Khosla *et al.*, 2001). It remains possible that estrogen might be involved in the striking difference of phenotypes between male and female mutants, suggesting the existence of crosstalk between ROS and estrogen signaling. Thus, one of the implications of our study is that antioxidant protection may be more important in women than in men.

In summary, our work shows that mitochondrial ROS is the driving force for accelerated aging and ROS impair the self-renewal of adult stem cells. The data suggest that one of the mechanisms of accelerated aging by ROS is through impairing the functions of adult stem cells. *Immp2l* mutation mouse model promises to be a very useful tool for the study of mitochondrial ROS in aging.

Experimental procedures

Animals

The generation of $Immp2l^{Tg(Tyr)979Ove}/Immp2l^{Tg(Tyr)979Ove}$ mutant mice has been described recently (Lu *et al.*, 2008). Mice were housed in the pathogen-free animal facility of Wake Forest University Health Sciences. Experiments were conducted in accordance with the National Research Council publication Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. Mice were kept in microisolator cages with 12-h light/dark cycles and were fed *ad libitum*. Genotypes of the mice were determined by coat color. Homozygous normal mice were albino because of the Friend virus B strain (FVB) background. Heterozygotes were slightly pigmented because of the expression of tyrosinase from one copy of the transgene. Homozygous mutant mice were darkly pigmented because of the expression of tyrosinase from both copies of the transgene.

Experimental design

Over 40 mice of each genotype and sex were produced for observation of age-associated disorders up to 30 months. Owing to the fact that mutant males and females are infertile, mutant and control mice were obtained by breeding between heterozygotes. At 2–3 month, 6 months, 10–12 months, and 20–22 months, 3–5 mice were sacrificed for tissue collection and histology analysis. After accidental death and euthanasia for tissue collection, the number of live animals at various ages is listed in Table 1. Most of the male mice were euthanized around the age of 10–12 months to study spermatogenesis in another study; thus, the number of male animals beyond this age was limited; *t*-tests (when comparing the means of two groups) and ANOVA analysis (when comparing the means of more than two groups and/or when the means are affected by two factors) were performed with software PRISM.

Histology and immunostaining

Tissues were fixed in Bouin's fixative (for testis) or 4% paraformaldehyde/PBS solution (for remaining tissues) at 4°C for overnight and were processed for paraffin embedding; 5- to 8-μm sections were obtained for hematoxylin and eosin staining and immunostaining. Rabbit anti-SOD1 (Santa Cruz, CA, USA) and monoclonal anti-SOD2 (Abcam, Cambridge, MA, USA) were used at 1:500 in antibody diluents (Dako North America, Inc., Carpinteria, CA, USA). Rabbit anti-HNE-protein adducts (Calbiochem, Gibbstown, NJ, USA) were used at 1:18000. After the incubation with primary antibody, biotin-conjugated anti-rabbit or anti-mouse secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were diluted at 1:300 and incubated with the sections at room temperature for 30 min. Then, the sections were incubated with HRP-conjugated streptavidin (Vector Laboratories) for 30 min at room temperature. Signals were visualized with AEC substrate kit (Vector Laboratories).

Table 1 Number of live animals at various ages

Age (month)	2	6–7	10–12	20–22	> 24
Control					
Female	40	35	25	15	10
Male	40	35	25	5	3
Mutant					
Female	40	35	25	15	10
Male	40	35	25	5	2

Images were acquired with an Axio M1 microscope equipped with an AxioCam MRc digital camera (Carl Zeiss, Thornwood, NY, USA). Different images were assembled into one file with Adobe Photoshop, with necessary resizing, rotation and cropping.

Food consumption

Mice were caged in groups or singly. Rodent chow diet (Prolab, RMH3000, with 22.5% protein, 52% carbohydrate, and 5.4% fat contributing 26%, 60%, and 14% calorie, respectively; total calorie 3.5 Kcal g^{-1}) and water were available *ad libitum*. Consumption of food was measured for each genotype weekly for a successive 4 weeks. Body weight of the animals was also monitored weekly. Food consumption was expressed as the ratio between daily food intake and body weight.

Wire hang assay

Control and mutant females of 21 months were placed on the top of a wire cage lid. The wire cage lid was shaken three times to cause the animal to grip the wires, and then, the lid was turned upside down. The latency to fall off the wire lid was quantitated. Latency over 60 s was recorded as 60 s. Three control and mutant mice were used to perform the test on 4 days. Each day three tests were performed and the average for each day was obtained.

ADSC cell isolation and differentiation

ADSC or vascular–stromal fraction cells from gonadal WAT were isolated from adult mice as previously described (Hauner *et al.*, 1987). Adipogenic differentiation was performed as described by Zuk *et al.* (Zuk *et al.*, 2001). Briefly, cells were plated at about 1×10^4 cells per cm^2 and cultured in growth medium (MEM-α with 10% FBS and antibiotics) until they reached 100% confluency, when the adipogenic differentiation medium was added. Adipogenic differentiation medium included dexamethasone (1 μM), 3–isobutyl-1-methylxanthine (500 μM), indomethacin (100 μM), and insulin (10 μg mL^{-1}) in growth medium. Medium was replaced every 3 days. Cells were stained for lipid vacuoles by Oil Red O 2 weeks after induction.

Isolation of murine bone marrow-derived stromal cells

Following sacrifice of the mice by CO_2 asphyxiation, femurs and tibia were dissected from the surrounding tissues. The epiphyseal growth plates were removed and the bone marrow was collected by flushing with MEM-α culture medium containing 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% fetal calf serum (FCS) with a 27-gauge needle. Single cell suspensions were prepared by gently mixing the cells with a pipette followed by filtration through a 70-μm strainer. Cells were centrifuged at 350 *g* for 10 min and the pellet was re-suspended in 5 mL of 1× Pharm Lyse buffer (BD Biosciences, Franklin Lakes, NJ, USA) and incubated for 10 min at room temperature. The cell suspension was centrifuged at 350 g for 10 min and the pellet was re-suspended in complete media for tissue culture in MEM-α medium containing 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10% FCS.

Colony formation assay

Fresh isolated ADSC cells were allowed to grow for 2–3 days in above-described growth medium, and 5000, 2500, and 1000 cells were plated in 6-well dishes. After 1 week of culture, the cells were fixed with methanol for 5 min at room temperature and stained for 30 min with Giemsa staining. The colonies were observed by microscopic examination.

Senescence-associated β-galactosidase staining

Cells were washed in PBS, fixed for 3–5 min at room temperature in 3% formaldehyde, washed, and incubated at 37°C (no CO_2) with fresh senescence-associated β-galactosidase solution as described previously (Dimri *et al.*, 1995).

Cell proliferation assay

The cells in growth medium were plated in 6-well plates at the density of 5×10^4cells per well. After reaching confluence, the cells were trypsinized and counted. A number of 5×10^4 cells were plated again for continued proliferation. Triplicate wells were used for cells from each animal. The assay was repeated on at least three pairs of animals (control and mutant) of the same age.

Glutathione (GSH) assay by monochlorobimane

Cells were trypsinized and equal numbers of cells were stained with 20 μM monochlorobimane (Invitrogen, Carlsbad, CA, USA) in PBS at 37°C for 30 mins. The cells were washed with and re-suspended in PBS before loading into black 96-well plate for fluorescence reading. Reading was performed with a Spectra-Max M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) at excitation of 355 nm and emission of 460 nm.

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Author contributions

Sunil George maintained the mouse colony and performed the major part of the experiments. Yan Jiao contributed to tissue collection and processing. Colin Bishop contributed to the characterization of the mutant, maintenance of the colony, and conceiving some of the experiments. Baisong Lu conceived and organized the project, performed experiments on live mice, analyzed the data, and wrote the paper.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Video S1 Ataxia of mutant mice.

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