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Model animals for the study of oxidative stress from complex II ☆

Takamasa Ishii [a], Masaki Miyazawa [a], Hiromi Onouchi [b], Kayo Yasuda [c], Phil S. Hartman [d], Naoaki Ishii [a,*]

[a] Department of Molecular Life Science, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
[b] Department of Ophthalmology, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
[c] Education and Research Support Center, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan
[d] Department of Biology, Texas Christian University, Fort Worth, TX 76129, USA

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ABSTRACT

Mitochondria play a role of energy production and produce intracellular reactive oxygen species (ROS), especially superoxide anion ($O_2^{\bullet-}$) as a byproduct of energy metabolism at the same time. $O_2^{\bullet-}$ is converted from oxygen and is overproduced by excessive electron leakage from the mitochondrial respiratory chain. It is well known that mitochondrial complexes I and III in the electron transport system are the major endogenous ROS sources. We have previously demonstrated that mutations in complex II can result in excessive ROS (specifically in SDHC: G71E in *Caenorhabditis elegans*, I71E in *Drosophila* and V69E in mouse). Moreover, this results in premature death in *C. elegans* and *Drosophila* as well as tumorigenesis in mouse embryonic fibroblast cells. In humans, it has been reported that mutations in SDHB, SDHC or SDHD, which are the subunits of mitochondrial complex II, often result in inherited head and neck paragangliomas (PGLs). Recently, we established *Tet-mev-1* conditional transgenic mice using our uniquely developed Tet-On/Off system, which can induce the mutated *SDHC* gene to be equally and competitively expressed compared to the endogenous wild-type *SDHC* gene. These mice experienced mitochondrial respiratory chain dysfunction that resulted in oxidative stress. The mitochondrial oxidative stress caused excessive apoptosis in several tissues leading to low-birth-weight infants and growth retardation during neonatal developmental phase in *Tet-mev-1* mice. *Tet-mev-1* mice also displayed precocious age-dependent corneal physiological changes, delayed corneal epithelialization, decreased corneal endothelial cells, thickened Descemet's membrane and thinning of parenchyma with corneal pathological dysfunctions such as keratitis, Fuchs' corneal dystrophy (FCD) and probably keratoconus after the normal development and growth phase. Here, we review the relationships between mitochondrial oxidative stress and phenomena in *mev-1* animal models with mitochondrial complex II SDHC mutations. This article is part of a Special Issue entitled: Respiratory complex II: Role in cellular physiology and disease.

1. Introduction

Energy metabolism in aerobic organisms is almost exclusively the result of glycolysis, the Krebs cycle and electron transport. With respect to electron transport, four membrane-bound complexes within mitochondria form the respiratory chain that sequentially transfers electrons through a series of donor/acceptors, with oxygen ($O_2^{\bullet-}$) as the final acceptor [1,2]. The eukaryotic mitochondrial electron transport system is composed of more than 80 subunits and requires more than 100 additional genes for its assembly [3].

The electron transport system or oxidative phosphorylation (OXPHOS) system is located within the mitochondrial inner membrane and is the major endogenous source of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) [4]. Several studies reported that exposing cells or tissues to hypoxia leads to an increase in mitochondrial ROS production, and that it is required for the cellular response to hypoxia [5–7]. Such endogenously generated molecules can readily attack a wide variety of cellular entities, resulting in damage that compromises cell integrity and function [8–10]. This can cause or at least contribute to a variety of pathologies, including some in humans [10–15]. It has been estimated that generation of $O_2^{\bullet-}$ and its dismutated product H_2O_2 through the action of superoxide dismutases (SODs; Cu/Zn-SOD, Mn-SOD) may constitute as much as 1-2% of total electron flow [16], although others have placed this value at 0.1% [17].

It is known that oxygen is initially converted to $O_2^{\bullet-}$ by electrons leaked from complexes I and mainly complex III [18–21]. Complex III generates $O_2^{\bullet-}$ by the auto-oxidation of ubisemiquinone (QH_o), formed during the Q cycle [22]. $O_2^{\bullet-}$ can be generated at two different sites, Q_o and Q_i, within complex III. The Q_o site releases $O_2^{\bullet-}$ into the intermembrane space, whereas the Q_i site releases $O_2^{\bullet-}$ into the matrix. Most of the $O_2^{\bullet-}$ generated by complex III is generated at the Q_o site [23]. In contrast, $O_2^{\bullet-}$ generated by complex I are probably

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* Corresponding author. Tel.: +81 463 93 1121x2650; fax: +81 463 94 8884.
E-mail address: nishii@is.icc.u-toaki.ac.jp (N. Ishii).

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released into the matrix. The mechanisms of $O_2^{\bullet-}$ generation in complex I are poorly understood, controversial and dependent on experimental conditions. Several studies revealed a much higher rate of $O_2^{\bullet-}$ production during reverse electron transport from succinate to NAD^+ than during forward electron transport [24].

In addition, it is shown that $O_2^{\bullet-}$ is also produced from complex II in *Saccharomyces cerevisiae* genetic backgrounds that compromise complex II functionality and from *Ascaris suum* mitochondrial complex II [25,26]. We have isolated an oxygen hyper-sensitive mutant, *mev-1*, from the nematode *Caenorhabditis elegans* [27]. We have found that the *mev-1* gene was a mitochondrial complex II SDHC [28] and $O_2^{\bullet-}$ is also produced from complex II [29]. We have hypothesized that high succinate concentrations outside the normal physiological range in mitochondrial or cytosolic energy metabolism might be important for $O_2^{\bullet-}$ production during reverse electron transport under hypoxic conditions [30,31]. In this paper, we review our studies using animal models, from nematodes to mice, with complex II SDHC mutations.

2. Lower eukaryote and animal models of mitochondrial complex II mutations

2.1. C. elegans mev-1 mutant with SDHC^{G71E} mutation

The electron transport system of *C. elegans* is composed of about 70 nuclear and 12 mitochondrial gene products. It closely parallels its mammalian counterpart in its metabolism and structure. *C. elegans* mitochondrial DNA (mtDNA) is similar in size and gene content to that of humans [32,33]. The *mev-1* mutant was isolated based upon its hypersensitivity to the ROS-generating chemical *me*thyl *v*iologen [27]. In addition to its methyl viologen hypersensitivity, *mev-1* mutants are oxygen hypersensitive with respect to both development and aging [27]. The *mev-1*(*kn-1*) mutation, which results in an amino acid substitution at the 71st position from glycine to glutamate (G71E), has been identified as residing in the putative gene *cyt-1* (a human *SDHC* gene homologue), which is homologous to the succinate dehydrogenase (SDH) cytochrome *b* large subunit in complex II [28]. The mutation results in a greater than 80% reduction in complex II activity in the mitochondrial membrane fraction. Complex II catalyzes electron transport from succinate to ubiquinone and contains the citric cycle enzyme succinate dehydrogenase (SDH), which is composed of the flavin protein (Fp), the iron-sulfur protein (Ip) and two other subunits (a small subunit of cytochrome *b* and a large subunit of cytochrome *b* encoded by *cyt-1*) [34–36]. *In vivo*, SDH is anchored to the inner membrane with cytochrome *b* and is the catalytic component of complex II. Using separate assays, it is possible to quantify specifically both SDH activity and complex II activity. This was done with wild type and *mev-1* after extracts of each were subjected to differential centrifugation to separate mitochondria and mitochondrial membranes from cytosol [28]. The SDH activity in the *mev-1* mitochondrial fraction was experimentally identical to that of wild type. As expected of a mitochondrial enzyme, no SDH activity was observed in the cytosol. Thus, the *mev-1* mutation affected neither SDH anchoring to the membrane nor SDH activity *per se*. However, it dramatically compromised the ability of complex II to participate in electron transport. The cytochrome *b* large subunit is also essential for electron transport to ubiquinone in complex III. Based upon its position, the mutation site in *mev-1* may affect the domain binding to ubiquinone.

The mean and maximum life spans of both the wild type and *mev-1* mutant were influenced by oxygen [37]. Wild-type life spans were not affected by oxygen concentrations between 2 and 40%. On the other hand, the mean and maximum life spans of the *mev-1* mutant under atmospheric conditions (21% oxygen) were shorter than wild type [37]. Fluorescent materials (lipofuscin) and protein carbonyl derivatives are formed *in vivo* as a result of metal-catalyzed oxidation and accumulate during aging in disparate model systems [38–41]. The presence of fluorescent materials and protein carbonyl modifications can be a specific indicator of oxidized lipid and protein. The *mev-1* mutants accumulated fluorescent materials and protein-carbonyl derivatives at significantly higher rates than did their wild-type cohorts [42,43]. Thus, the aging process in *mev-1* animals approximates that of wild type except for its precocious nature.

The biochemical pathologies of *mev-1* include elevated ROS. Specificity, $O_2^{\bullet-}$ levels in both intact mitochondria and sub-mitochondrial particles are approximately two times greater in *mev-1* mutants as compared to wild type [29]. Given that most $O_2^{\bullet-}$ generation is thought to occur around complex III, this means that the *mev-1* mutation either exacerbates $O_2^{\bullet-}$ production at this location or, in some indirect way, increases $O_2^{\bullet-}$ production at another point in electron transport, perhaps even at complex II. Several experiments suggest the latter. Another of the biochemical pathologies is that of reduced glutathione concentration in *mev-1* animals [29]. The *mev-1* mutation also caused supernumerary embryonic apoptosis especially under hyperoxia [44]. The abnormal apoptosis was suppressed by mutations in either *ced-3* or *ced-4*, indicating that the inappropriate signal in *mev-1* embryos stimulated induction of the normal *ced-9*/*ced-3*/*ced-4* apoptotic pathway in *C. elegans* [44]. Furthermore, the *mev-1;ced-3* double mutant lived longer than *mev-1*, which suggests that the supernumerary apoptosis contributed to the phenotype of life shortening in *mev-1* [44]. In addition, the oxidative stress by hyperoxia in *mev-1* animals rendered them hypermutable to nuclear mutations [45]. Finally, a number of biochemical pathologies likely derive from the role played by succinate dehydrogenase in the citric cycle. First, the ratio of lactate to pyruvate is significantly higher in *mev-1* mutants, suggesting that a metabolic imbalance known as lactate acidosis occurs in these animals. Second, a number of citric cycle intermediates are present at abnormal concentrations in *mev-1* mutants. Conversely, ATP levels are normal in *mev-1* mutants. This was initially surprising but may suggest that *mev-1* animals rely more heavily on glycolysis for energy acquisition, thus explaining the elevated lactate levels. However, it is also possible that ATP consumption is decreased in *mev-1* because of some sort of global decrease in the metabolic rate that acts to counterbalance the compromised ATP generation in *mev-1* [29]. These results suggest that age-related complex II deterioration might also produce $O_2^{\bullet-}$ and consequently accelerate aging.

In a similar fashion, Dr. Lemire and colleagues constructed transgenic *C. elegans* strains with a series of mutations in the succinate dehydrogenase iron-sulfur subunit (SDHB-1) (a human SDHB homologue). They also resulted in reduced life spans [46]. These strains are also more sensitive to oxygen and paraquat (methyl viologen). They overproduced superoxide anion with decreased succinate–cytochrome *c* reductase activity compared to the control strain. Thus, they recapitulate the phenotypes of the *mev-1* mutant.

2.2. Drosophila mev-1-mimic and another complex II mutated transgenic mutants

We investigated the phenotypic effects of mutation in the *sdhC* gene (a human *SDHC* gene homologue) encoding succinate dehydrogenase C in *Drosophila* by using transgenic flies expressing a dominant-negative form, SdhC^{I71E} [47]. Expression of SdhC^{I71E} significantly reduced the mean lifespan by 22% compared to that of control flies [47]. The amount of protein carbonyl was significantly increased, suggesting that a high level of oxidative stress was induced in these flies with decreasing of complex II activity [47].

Furthermore, Dr. Benzer and colleagues performed a genetic screen for mutations that cause decreased survival under hyperoxia (100% O_2) [48]. Among a collection of P-element insertion mutants [49], they identified a hyperoxia-sensitive line, EY12081, which has an insertion in the *sdhB* gene (a human *SDHB* gene homologue) encoding the iron-sulfur protein (Ip, SDHB) as one of mitochondrial complex II subunits. The

SDHB subunit contains three iron-sulfur centers that are speculated to be important for mediating that function [34–36]. Under hyperoxia, the mean survival time of *sdhB*^{EY12081} flies, which have the P-element insertion site within the 5′ untranslated region (UTR) of the gene resulting in reducing the expression of *sdhB* gene, was reduced to 10% of that of normal flies [48]. Under normoxic conditions, *sdhB*^{EY12081} flies displayed a 66% decrease in mean survival time and a 17% decrease in maximum survival time. There was a 56% decrease in the complex II-specific (succinate-dependent/antimycin A-sensitive) respiration rate, a 40% decrease in the complex II-mediated electron transfer ratio (malonate-sensitive succinate–cytochrome *c* reductase activity) and a 32% increase in mitochondrial hydrogen peroxide production, as compared with wild-type controls [48]. Both mutants (SdhC^{I71E} and *sdhB*^{EY12081}) mimic the nematode mutants; namely, they are very similar in complex II mutation-associated ROS production, highly sensitive to oxidative stress, including hyperoxia, and age precociously. The results from these lower animal models support the consequential damages of ROS production with electron leakage by mitochondrial complex II deficiency, which ultimately lead to accelerated aging and age-associated diseases.

3. Human mitochondrial complex II mutations and diseases

In humans, succinate dehydrogenase (SDH) is an enzyme complex II composed of four subunits encoded by four nuclear genes (*SDHA*, *SDHB*, *SDHC* and *SDHD*). SDHC (cybL, 15 kDA, 169 amino acids) and SDHD (cybS, 12 kDa, 159 amino acids) subunits are hydrophobic and provide the membrane anchor and binding site for ubiquinone. SDHA (flavoprotein, 70 kDa, 664 amino acids) and SDHB (iron–sulphur protein, 27 kDa, 280 amino acids) are probably hydrophilic with the former involved in substrate binding and oxidation and the latter in electron transfer [34–36]. The crystal structure of mitochondrial respiratory membrane protein complex II has been elucidated in porcines [50]. The *SDHB* (35.4 kb, 8 exons) and the *SDHC* (50.3 kb, 6 exons) genes are located on the short and long arms, respectively, of chromosome 1 (1p36 and 1q23.3). The *SDHD* gene is located on 11q23.1, spans 8.9 kb and contains four exons whilst *SDHA* lies on the short arm of chromosome 5 (5p15) and is composed of 15 exons spread over a genomic region of 38.4 kb. Homozygous germline mutations affecting the *SDHA* gene cause Leigh syndrome, a sub-acute necrotizing encephalomyelopathy during infancy [51,52]. *SDHD*, *SDHB* and *SDHC* heterozygous mutations cause a genetic predisposition to non-chromaffin palagamgliomas (PGLs) and adrenal/extra-adrenal pheochromocytomas (PHEOs) [53–55] called 'PGL/PHEO syndrome. PGLs are usually benign and slow-growing tumors of the parasympathetic ganglia with an incidence of roughly 1:30,000–1:100,000 in the general population. They are more frequently located in the head and neck region (HNPGLs) at the carotid bifurcation (carotid body tumor), along the vagal nerve, in the jugular foramen and in the middle ear space. Following the discovery of SDHD (OMIM ID: 602690) as the gene responsible for PGL1 in familial HNPGLs [53], it was subsequently recognized that two other subunits of this mitochondrial enzyme, SDHC (PGL3, OMIM ID: 602413) and SDHB (PGL4, OMIM ID: 185470) were associated with heritable PHEO and/or PGL [54,55]. *SDHB*, *SDHC* and *SDHD* gene mutations are responsible for 6% and 9% of sporadic PGLs and PHEOs, respectively. They also constitute 29% of pediatric cases, 38% of malignant tumors and more than 80% of familial aggregations of PHEO and PGL [56]. Known risk factors for HNPGLs include conditions associated with chronic hypoxia such as living at a high altitude and respiratory or heart diseases with chronic arterial hypoxemia. In fact, Dr. Gimenez-Roqueplo and colleagues studied the biological effects of a loss-of-function *SDHD* germline mutation (p.Arg22X) in an extra-adrenal PGL and a missense *SDHB* germline mutation (p.Arg46Gln) in a malignant PHEO with somatic terminal deletion of 1p [57,58]. In tumor tissues, SDH activity was abolished with increased expression of the hypoxia inducible factors (HIF) 1alpha and 2alpha. In addition, expression of vascular endothelial growth factors (VEGFs) associated with VEGF-R1 and VEGF-R2 were increased in endothelial cells. This is in agreement with the high vascularization of this endocrine tumor [57,58]. In addition, the inactivation of SDH activity and succinate accumulation was shown to inhibit prolyl-hydroxylation of HIF1a and HIF2a, which is an essential step for its degradation through the complex VHL-ElonginD-C-Cul2 [59]. We speculate that SDH deficiency might trigger hypoxic conditions, resulting in increased activity of HIFs. Collectively, these alterations may trigger changes in cellular metabolism, angiogenesis, metastasis and cell proliferation [60].

4. Comparisons of SDHC between prokaryotes, lower eukaryotes, mouse, pig and human

The amino acid sequences of *Escherichia coli* K-12 (GenBank ID: AAA23893.1), *C. elegans* (GenBank ID: AAA20081.1), *Drosophila melanogaster* (fruit fly) (GenBank ID: AAF54602.2), *Mus musculus* (house mouse) (GenBank ID: AAH05779.1), *Sus scrofa* (pig) (UniProtKB/Swiss-Prot ID: D0VWV4.2) and *Homo sapiens* (human) (GenBank ID: AAC27993.1) for the gene named succinate dehydrogenase C (SdhC), cytochrome *b* large (CybL), CYT-1 or SDHC subunit were retrieved from Entrez Nucleotide of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein). These amino acid sequences can be analyzed by the multiple sequence alignment tools of ClustalW [61], which is one of several mainstream, public-access sequence analysis applications provided by the European Bioinformatics Institute (EMBL-EBI) (Fig. 1) [62]. The sequence analysis can be also performed by InterProScan, which was created to unite secondary databases that contain overlapping information on protein families, domains and functional sites [63]. InterProScan is also provided by the European Bioinformatics Institute (EMBL-EBI). Consensus patterns in the PROSITE database [64]: R–P–[LIVMT]–x(3)–[LIVM]–x(6)–[LIVMWPK]–x(4)–S–x(2)–H–R–x–[ST], which is a putative a heme ligand "succinate dehydrogenase cytochrome b subunit signature 1" (IPR018495, SDH_CYT_1, PS01000) and H–x(3)–[GA]–[LIVMT]–R–[HF]–[LIVMF]–x–[FYWM]–D–x–[GVA], which is a putative heme ligand "succinate dehydrogenase cytochrome b subunit signature 2" (IPR018495, SDH_CYT_2, PS01001) are highly conserved among animals (Fig. 2) [65–76]. The SDH_CYT_1 domain sequence is positively related to the activity of succinate–ubiquinone oxidoreductase as a ubiquinone-binding site [68,69,72–76].

In the *C. elegans mev-1* mutant, the mutational site which has been located in glycine at 71st amino acid position in the ubiquinone-binding region between succinate–ubiquinone oxidoreductase activity region is maintained at the neutral and hydrophobic amino acid residues between *Drosophila* (Isoleucine at the 71st amino acid position: I71), pig and human (Isoleucine at the 69th amino acid position: I69) and mouse (Valine at the 69th amino acid position: V69). Therefore, it was anticipated that this mutation, which was converted from a neutral to an acidic amino acid, could be applied to higher mammals for use as models to study aging and age-associated diseases that are triggered by oxidative stress.

In humans, it was reported that the *SDHC* gene mutation caused paraganglioma type 3 (PGL3) [54]. Since that time, several type mutations of SDHC have been found in paragangliomas, pheochromocytomas and gastrointestinal stromas (Table 1). Most of the *SDHC* gene mutations resulting in amino acid missense, flame shift, nonsense or deletion mutations destroy or delete the quinone-binding site (from 50 to 74 amino acid sequence) or heme-binding site (from 127 to 140 amino acid sequence) in the succinate–ubiquinone oxidoreductase main activity region (Table 1). However, there are some mutations in the N terminal region from the 1st to 41st amino acids that are not in *E. coli*, so the structure and function have not been become clear in mammals (Fig. 1). Using the multiple sequence alignment tool ClustalW, a new predicted quinone or heme binding amino acid

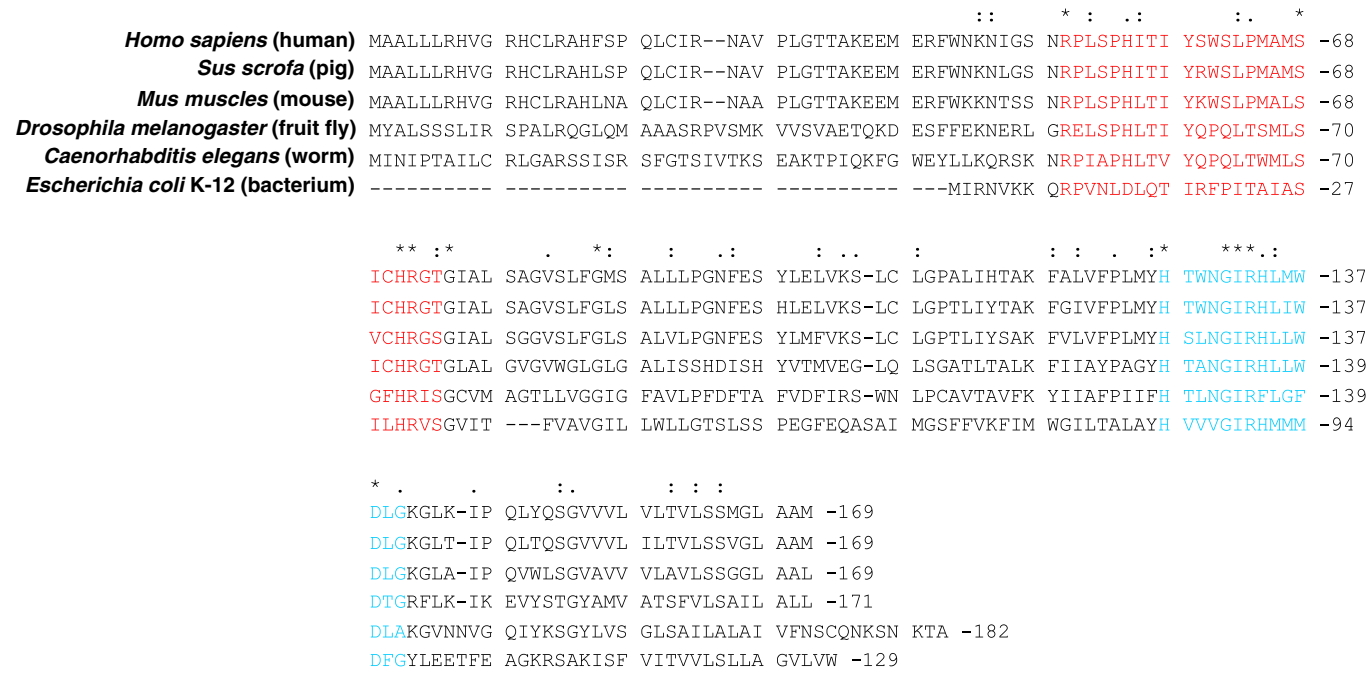

Fig. 1. The alignment between SDHC amino acid sequences of *Escherichia coli* K-12, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus muscles*, *Sus scrofa* and *Homo sapiens*. Red color and blue color indicate the SDH_CYT_1 domain and SDH_CYT_2 domain regions. Asterisk (*) indicates positions which have a single, fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties — scoring > 0.5 in the Gonnet PAM 250 matrix. Period (.) indicates conservation between groups of weakly similar properties — scoring = <0.5 in the Gonnet PAM 250 matrix.

consensus sequence, which is a reversed putative heme ligand "Succinate hehydrogtenase cytochrome b subunit signature 1" (IPR018495, SDH_CYT_1, PS01000) (R–P–[LIVMT]–x(3)–[LIVM]–x(6)–[LIVMWPK]–x(4)–S–x(2)–H–R–x–[ST]), can be found from the 5th to 30th amino acids in the N terminal region of SDHC (Fig. 2). The sequence contains some well-known heme-binding motifs, including nucleophilic amino acids such as cysteine, histidine and serine, basic amino acids and hydrophobic amino acids. Therefore, we expected that the mutations in the N terminal region of SDHC should also result in tumorigenesis which causes paragangliomas, pheochromocytes and gastrointestinal stromas.

Based upon these findings, we anticipated that the *C. elegans mev-1*-type mutation, which resulted in the missense amino acid mutation to glutamate, could be useful in clarifying not only the effects of oxidative stress on aging but also tumorigenesis and other age-related pathologies in mouse models.

5. Mouse embryonic fibroblast cells with SDHC^{V69E} mutation

We established a transgenic mouse embryonic fibroblast NIH3T3 cell line with the equivalent mutation (V69E) in SDHC as *C. elegans mev-1* [77]. The mutation corresponds to the amino acid substitution of glutamic acid from glycine at position 71 (G71E) in the *C. elegans mev-1* mutant allele *kn1* [28]. It is thought that the serine, histidine and cysteine residues, which are located in the closed mutation site within the ubiquinone binding region, constitute an active center of

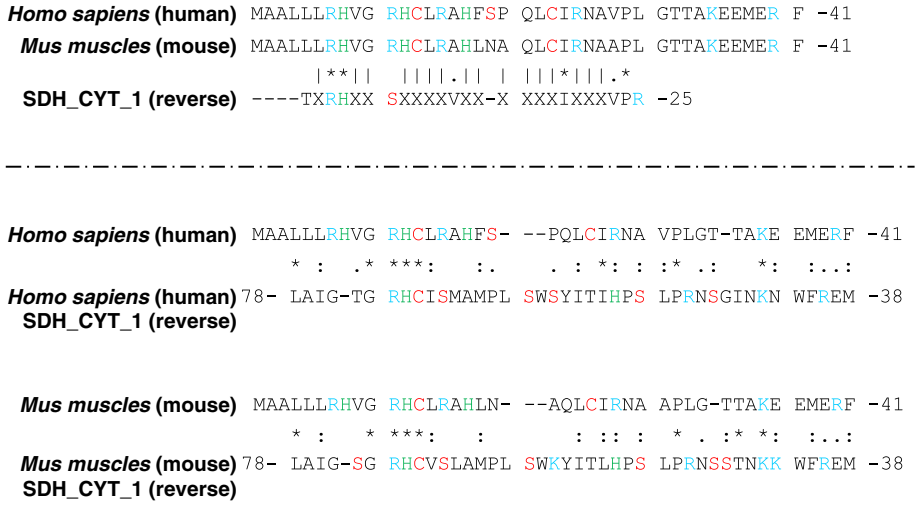

Fig. 2. The alignments between SDHC N-terminal regions of *Mus muscles* and *Homo sapience*, and reverse SDH_CYT_1 domain sequence. Red color, green color and blue color indicate the nucleophilic amino acids, nucleophilic basic amino acids and basic amino acids. Asterisk (*) indicates positions which have a single, fully conserved residue. Colon (:) indicates conservation between groups of strongly similar properties — scoring > 0.5 in the Gonnet PAM 250 matrix. Period (.) indicates conservation between groups of weakly similar properties — scoring = <0.5 in the Gonnet PAM 250 matrix.

Table 1
Cases of *SDHC* gene mutations in human diseases.

Case reports	Exon	Mutation (nucleotide)	Mutation (codon and amino acid)	Source
1	1	3G>A	M1I/ loss of the start codon/ M38 available for new start codon/ exon 1 and 2 deletion	Niemann S. et al. [54], *Science* (2000)
2	Intron 5	IVS5+1G>T	Exon 5 skipping deletion/ reading-frame shift of exon 6/ heme binding site deletion	Niemann S. et al. [100], *Hum Genet* (2003)
3	6	473T>C	L158P/ exon 6 terminal deletion	Bauters C. et al. [101], *J Med Genet* (2003)
4	6	8372 bp deletion	Exon 6 deletion/ heme binding site deletion	Baysal B.E. et al. [102], *J Med Genet* (2004)
5	1	1A>G	M1V/ similar to case report #1	Schiavi F. et al. [103], *JAMA* (2005)
	2	39C>A	C13X/ exon 2 terminal deletion	
	4*	214C>T	R72C/ quinone binding site mutation	* Bayley J.P. et al. [104], *BMC Med Genet* (2006)
	6	439C>T	Q147X/ exon 6 terminal deletion	
6	3	126 G/A	W42X/ exon 3 terminal deletion	Mannelli M. et al. [105], *J Med Genet* (2007)
7	Intron 5	IVS5+1G>A	Similar to case report #2	McWhinney S.R. et al. [106], *N Engl J Med* (2007)
	2	43C>T	R15X/ exon 2 terminal deletion	
8	5	397C>T	R133X/ exon 6 deletion/ heme binding site deletion	Zbuk K.M. et al. [107], *Nat Clin Pract Oncol* (2007)
9	Intron 2	78-2A>G	Exon 3 skipping deletion	Peczkowska M. et al. [108], *Nat Clin Pract Endocrinol Metab* (2008)

oxidoreductase and acts as nucleophilic amino acid. The mutation probably results in an excess electron leakage from electron transport by decreasing the affinity between complex II and ubiquinone and thereby uncouples electron transfer. This alternation would be predicted to reduce complex II-III activity.

The mutation at the 69th position, changing a neutral amino acid (valine) to an acidic amino acid (glutamate) in mouse SDHC, is located within the functional ubiquinone-binding region of complex II [77]. After transfection, we selected SDHC E69 cell lines as *mev-1* cells that expressed equal amounts of sdhc mRNA from the transgene and endogenous wild-type allele [77]. Overexpressed transgene cell lines were not obtained, most likely because cells with more than 80% abnormal mitochondrial DNA are inviable [78]. Consistent with this prediction, RNAi with *cyt-1* produced an embryonic lethal in *C. elegans* [79].

$O_2^{\bullet-}$ production was slightly but not statistically significantly higher in untreated mitochondria isolated from *mev-1* cells. The addition of succinate (a substrate for complex II that stimulates complex II activity) resulted in a large increase in $O_2^{\bullet-}$ production in mitochondria isolated from the *mev-1* cells. The enzymatic activity of complex II in the *mev-1* cells was reduced to 40% whereas the activity of complex I was unaffected [77]. ATP levels were not affected, suggesting that this mutation did not directly compromise cell survival through reduced respiration *per se*. Under these conditions, $O_2^{\bullet-}$ levels were significantly higher in intact mitochondria isolated from *mev-1* cells at both one month and three months after establishment [77].

The *mev-1* cells accumulated cytoplasmic carbonyl proteins, a marker of oxidative stress, at a faster rate than wild type. In addition, the amount of 8-hydroxydeoxyguanosine (8-OHdG), a DNA marker of oxidative stress was two-fold higher in *mev-1* cells [77]. During the time necessary for colony formation on the medium plates, wild-type NIH3T3 cells maintained normal fibroblast morphology and grew in a monolayer. Conversely, the *mev-1* cells showed a loss of contact inhibition and had many apoptotic molecule-like granules during the first month after establishment [77]. During the period of colony formation, some clefts characteristic of cell death were found in the center of some colonies. Consistent with this, the activity of the apoptosis marker caspase 3 was 1.3 to 1.8 times higher in *mev-1* cells [77]. In three-month *mev-1* cells, the morphology was changed from the typical solid and elongated fibroblasts to smooth and rounded cells. In addition, the *mev-1* cells formed multiple layers. The doubling time of one-month *mev-1* cells after establishment was 1.5 to 2 times slower than that of wild-type cells; however, in three-month *mev-1* cells the doubling time was completely recovered to that of wild type [77].

When one-month *mev-1* cells were injected under the epithelium of nude mice, they rapidly disappeared as compared to wild type. This suggests that these cells were dying of apoptosis and were phagocytized shortly after injection. Conversely, injecting the same number of three-month *mev-1* cells resulted in the production of tumors [77,80]. The transformation rate on soft-agar medium for wild-type NIH3T3 cells was less than 1×10^{-6}. On the other hand, the rates were 5×10^{-4} for the one-month *mev-1* cells and 5×10^{-3} for the three-month cells [77]. Thus, the *mev-1* cells had 100- to 1,000-fold higher transformation rates than wild-type cells. The 6-thioguanine tolerance test was performed as an indicator of mutations in the hprt gene on nuclear DNA [77]. The three-month *mev-1* cells were approximately twice as resistant as the one-month and wild-type cells, indicating that *mev-1* cells are hypermutable with excessive apoptotic cell death [77]. In the transformed three-month *mev-1* cells, benign tumor's characterizations were confirmed under the epithelium of nude mice [80]. Interestingly, the cells maintained active caspase 3 through caspase 8 and 9 with cytochrome *c* release from mitochondria and p53 and p21 activation [80]. On the other hand, cell proliferation was activated by AP-1 transcriptional activation through Ras–Raf and Ras–MEKK signal transductions [80]. Therefore, we anticipate that the *mev-1*-like cell lines are suitable models to clarify the molecular mechanisms of cell senescence and tumorigenesis and the pathogenesis of paragangliomas, pheochromocytes and gastrointestinal stromas in humans (Fig. 3).

6. *Tet-mev-1* conditional transgenic mice with $SDHC^{V69E}$ mutation

6.1. Establishment of mev-1-mimic (Tet-mev-1) conditional transgenic mice

A *mev-1* transgenic mouse was created that contained the mutated $SDHC^{V69E}$ transgene (unpublished data). This mouse had increased $O_2^{\bullet-}$ levels in the mitochondria of its heart and muscle as well as decreased body weight and locomotion activity. Abnormal mitochondrial structures were observed, especially in muscles. These manifested themselves as swelling and enlargement, which resulted in muscle-fiber atrophy. Unfortunately, this *mev-1* transgenic mouse was infertile, which prevented propagation of the strain for further studies. Additional conditional transgenic mice that ubiquitously induce the mutated $SDHC^{V69E}$ over-expression are extremely likely to meet with a similar fate, significantly limiting their utility. In order to overcome this limitation we established a *mev-1* conditional transgenic mouse that does not constitutively overexpress the mutated $SDHC^{V69E}$ encoding *SDHC* transgene [81]. We contemplated developing another *mev-1* model mouse using a knock-in approach. However, it has been reported that most of hereditary diseases and genetic disorders have been impacted by genomic imprinting [82,83]. Therefore, it was feared that the individual phenotypes could be unstable in the same strain, even though it has been unclear whether the imprinting

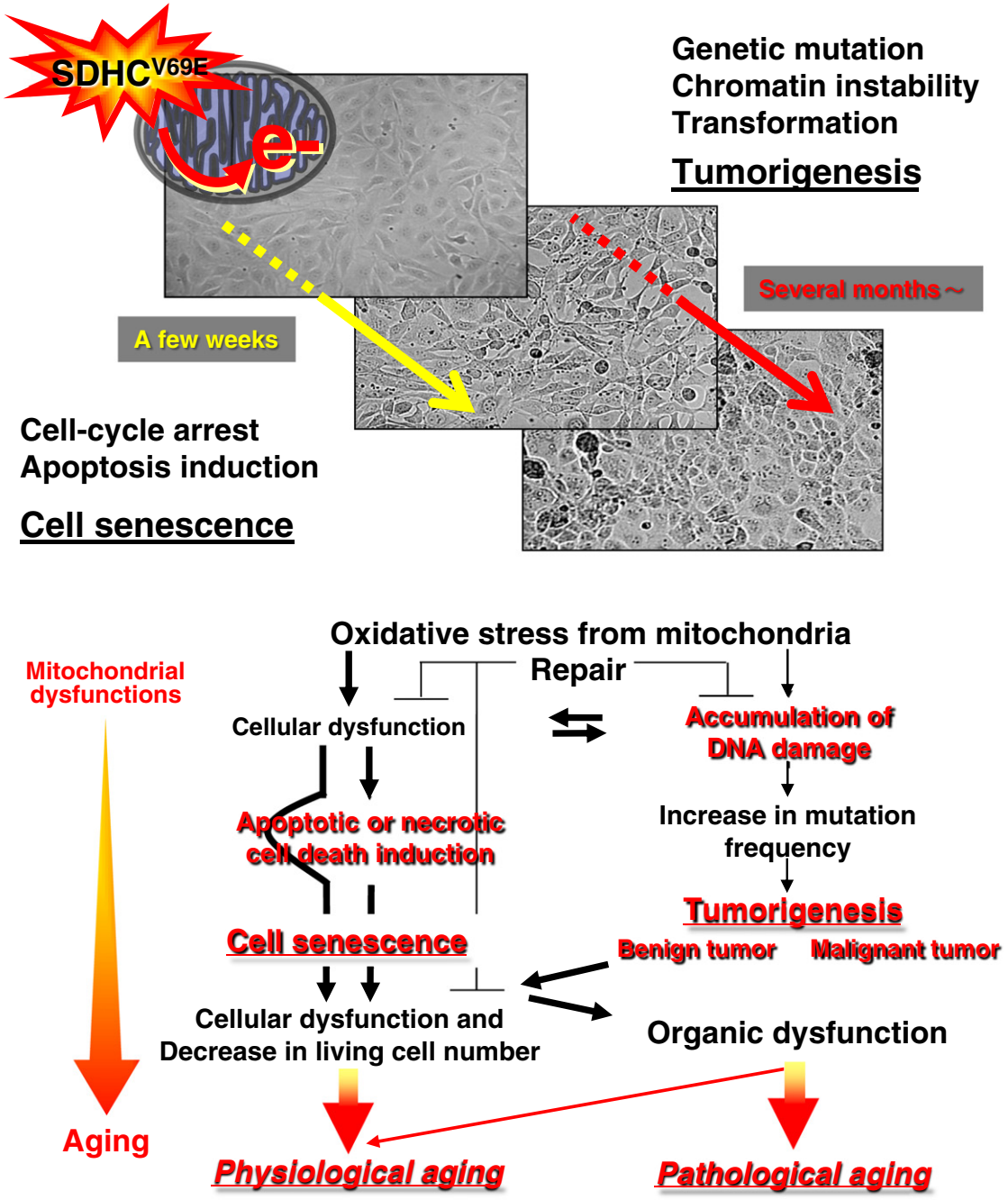

Fig. 3. Schematic of phenotypic alterations in *mev-1* animal models with aging. Pictured at the top is the progression in morphological changes as *mev-1* cells are cultured. The bottom portion indicates the progressive effects of mitochondrial dysfunction, which result ultimately in physiological and pathological aging.

occurs at the *mev-1*-locus (1q23.3). Given this, we opted to employ a modified tetracycline system (Tet-On/Off system) to construct a *mev-1*-mimic mouse model.

The Tet-On/Off system allows for the induction and repression of transgenes in which expression is reversibly controlled by the presence of the antibiotic tetracycline [or more commonly the tetracycline-derivative doxycycline (Dox)]. As developed by Bujard and coworkers [84], the original system employed a tetracycline transactivator (tTA) protein that was created by fusing two proteins (TetR from *E. coli* and VP16 from Herpes Simplex Virus). The original Tet-On/Off system was modified using the reverse Tc-controlled transactivator (rtTA) [85] and the Tc-controlled transcriptional silencer (tTS) with the improved *Kruppel*-associated box (KRAB) domain of human Kox-1 [86,87] that also included a nuclear localization signal (nls). In this system, the tTS binds to the Tc-responsive element (tetracycline-responsive element: TRE) in the absence of Dox, leading to the repression of leaky activation of TRE-mediated transcription caused by weak binding of rtTA to TRE. As the result of these modifications, our modified Tet-On/Off system enables the transgene to be expressed at lower levels such that they more reasonably approximate endogenous expression of the transgene. This was ultimately engineered into a single plasmid vector rather than the three-plasmid system typical of Tet-On/Off systems [81]. It is also not leaky in the absence of the inducer Dox, which can be problematic at times.

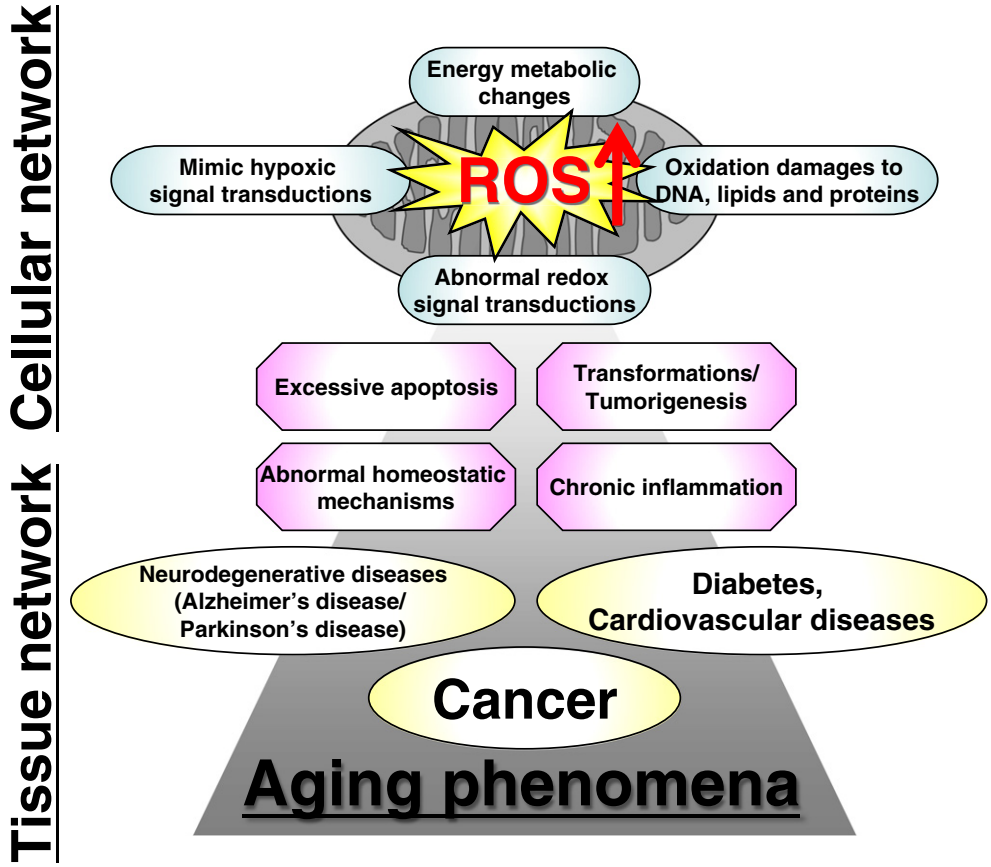

Fig. 4. Mitochondrial ROS, cellular dysfunctions, organismal dysfunctions, and aging phenomena. This figure summarizes effects of elevated ROS in mitochondria, as it occurs in *mev-1* model systems, and the various consequences of excess ROS on a variety of phenomena that relate to aging.

6.2. Phenotypes in the cell growth activation stage "neonatal development"

The fourteen lines of *mev-1*-mimic (*Tet-mev-1*) conditional transgenic mice with SDHC^{V69E} mutation were established using our modified Tet-On/Off construct [81]. As was the case with *C. elegans mev-1* and mice embryonic fibroblast *mev-1* cells, these mice were all hypersensitive to *m*ethyl *v*iologen, which induces oxidative stress [81]. In newborn *Tet-mev-1* mice that had been exposed continuously to Dox from the embryonic stage onward resulting in the low-birth-weight infants, $O_2^{\bullet-}$ levels were increased compared to those of wild-type mice treated with Dox [81]. Carbonylated proteins accumulated to higher levels in the intracellular membrane fraction protein of newborn *Tet-mev-1* mice that were exposed to Dox. Moreover, both TUNEL-stained and c-Caspase-3 immunostained brown cells, which are markers of programmed cell death, were observed more frequently in *Tet-mev-1* mice than in the wild-type controls. This included brain, lung, liver, kidney (especially in the adrenal region), salivary grand, nasal sinus tissue (especially secretory cells and mucosal cells) and muscles [81]. Interestingly, these programed cell deaths were observed in the stomach, intestine, spleen and lymphatic tissues of wild-type C57BL/6j mice with Dox and *Tet-mev-1* mice with Dox at roughly equal rates. Thus, the increased mitochondrial oxidative stress that led to excessive apoptosis resulted in a significant decrease in body size and weight between the developmental stages leading to the growth retardation in *Tet-mev-1* mice, just as in the *C. elegans mev-1* mutant [81]. In *Tet-mev-1* treated with Dox, the electron transport ratio from complex II to III through ubiquinone was decreased as compared to the wild-type C57BL/6j treated with Dox. Surprisingly, there was no significant difference in the reduced cytochrome *c* levels dependent on succinate–ubiquinone oxidoreductase activity as was the case with malonate-sensitive succinate–cytochrome *c* reductase activity [81]. We have considered that the methodology may be not be sufficiently optimized to analyze the activity in lysed samples of developmental stage tissues.

These phenomena, including decreasing electron transport ratio, increase in $O_2^{\bullet-}$ production and accumulation levels, excessive apoptosis, low-birth-weight infants and growth retardation in *Tet-mev-1* mice, were recovered by CoQ-H_2 supplementation to that of the wild-type C57BL/6j [81]. This suggests that the electron leakage led to $O_2^{\bullet-}$ overproduction from mitochondria with excessive apoptosis. In turn, these biochemical defects resulted in the low-birth-weight infant and growth retardation during the embryonic and neonatal periods, which was caused by a decrease in the affinity of ubiquinone for complex II with mutated SDHC^{V69E}.

6.3. Phenotypes in the matured tissue "cornea" after development

We have determined the tissue functional changes in response to mitochondrial oxidative stress in C57BL/6j mice [88]. We determined that the oculus was more sensitive to mitochondrial oxidative stress with age compared to other tissues. Therefore, we expected that the oculus, which has contact with atmosphere, acutely manifested the oxidative stress-induced *mev-1*-mimic phenotypes. In fact, a preponderance of cataracts was demonstrated in *mev-1* transgenic mouse (unpublished data).

In the eyes of *Tet-mev-1* conditional transgenic mouse, complex II-III activity was decreased. This was likely caused by the electron leakage between complex II and ubiquinone when the mutant SDHC^{V69E} was induced by doxycycline treatment [89]. This led to increased levels of carbonylated protein and 8-OHdG nucleotide levels with increasing mitochondrial superoxide anion ($O_2^{\bullet-}$) production in the eyes, particularly as *Tet-mev-1* mice grew older. In the corneal epithelium of

Tet-mev-1 mice, the proliferation of epithelial basal cells was decreased, resulting in delayed epithelialization with keratitis relative to wild-type C57BL/6j mice, particularly as animals aged [89]. It has been reported that keratitis and delayed epithelialization might be caused by hyperglycemia and high oxidative stress conditions [90]. In the corneal endothelium of *Tet-mev-1* mice, it was confirmed that the age-dependent decrease in cell number was accelerated compared to wild-type C57BL/6j mice [89]. Interestingly, the thickened Descemet's membrane with age was present in *Tet-mev-1* mice relative to wild-type C57BL/6j mice. Both the decrease of corneal endothelial cells and the increase in thickness of Descemet's membrane in *Tet-mev-1* mice are consistent with the pathological phenotypes of Fuchs' corneal dystrophy (FCD) [91]. Finally, corneas were thinner, primarily owing to decreased numbers of corneal stromal cells as the *Tet-mev-1* animals aged. As well, increased catalase activity in *Tet-mev-1* mice was noted compared with wild-type C57BL/6j mice [89]. It has been reported that the catalase activity is increased in human keratoconus [92]. Our results suggest that the pathogenesis of keratoconus was due to corneal thinning with decreasing number of corneal stromal cells caused by excessive mitochondrial oxidative stress with increasing catalase activity.

To summarize, we have demonstrated accelerated age-dependent corneal pathophysiological changes in a *Tet-mev-1* mouse model with excessive mitochondrial oxidative stress. This includes delayed epithelialization with keratitis, decreasing endothelial cell number, thickened Descemet's membrane and corneal stromal thinning. Therefore, we anticipate that the pathogenesis and molecular mechanisms of FCD, delayed epithelialization with keratitis and keratoconus can be clarified by future analyses of *Tet-mev-1* mice with mitochondrial oxidative stress. In recent years, ROS have been increasingly implicated as a causative factor triggering eyes disease; however, a specific role and molecular mechanisms by which ROS effect ocular dysfunctions (dry eyes, glaucoma and age-related macular degeneration *etc.*) remain elusive [93–95]. In the future, we anticipate that these pathologies might be clarified by analyses of *Tet-mev-1* mice.

7. Future directions

Chronic elevation in ROS levels presumably results in damage to the various components of the electron transport system, which in turn results in the production of ROS at an even higher rate. The net result of this cascade is cellular and organismal aging (Figs. 3 and 4). Oberley and colleagues argued for the importance of $O_2^{\bullet-}$ in cancer, differentiation and aging [96–98]. Our data imply that a mutation in the *mev-1* (corresponding to *cyt-1* or SDHC) gene of both *C. elegans* and mouse cells leads to apoptosis and high mutation frequency in the nuclear genome, most likely because mitochondrial abnormalities lead to excess ROS production in mitochondria. Mitochondrially derived ROS can mutate other genes, including tumor suppressor genes and oncogenes and can lead to cellular transformation. Indeed, a significant fraction of the *mev-1* cells that survived apoptosis were transformed. These data support the notion that oxidative stress from mitochondria play an important effect on both apoptosis, which leads to precocious aging and tumorigenesis (Table 2; Figs. 3 and 4).

A mutation in SDHB, SDHC or SDHD of complex II was found in patients of PGL/PHEO syndromes [53–58]. It is still unclear whether oxidative stress contributes to the symptoms of these diseases, but in general, inhibition of electron flow causes electron leakage from the complexes and consequently increases ROS production. It has been hypothesized that reverse electron transport under hypoxic conditions or excessive succinate overload causes the ROS production leading to a series of hypoxic responses that include transcriptional alterations leading to changes in cellular metabolism, angiogenesis, metastasis and cell proliferation that could result in tumorigenesis [59,60,99] (Fig. 3).

In addition, it is well known that oxidative stress with mitochondrial dysfunctions can result in the breakdown of homeostasis functions and tissue networks leading to hyperglycemia and chronic inflammation. These can contribute to neurodegenerative diseases, cardiovascular diseases and diabetes [12–15] (Fig. 4). Thus, we anticipate that the *mev-1* models cause an electron leakage resulting in $O_2^{\bullet-}$ production between complex II and ubiquinone. As such, the *Tet-mev-1* mice with $SDHC^{V69E}$ mutation will be especially suitable for not only for the study of tumorigenesis and carcinogenesis such as PGL/PHEO syndromes, but also some age-related disease models. These would include chronic inflammation-related diseases or lifestyle-related diseases such as cardiovascular disease and diabetes as well as excessive apoptosis-related diseases; that is, neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Fig. 4).

We have recently analyzed a variety of additional *Tet-mev-1* mouse phenotypes. The data would suggest that these *mev-1* animals

Table 2
Functional changes of *mev-1* models with SDHC mutation.

Functional changes	References
Mitochondrial structural abnormalities	
Disappearance of cristae	Senoo-Matsuda N. et al. [44], *J Biol Chem* (2003)
Swellings and enlargements	Ishii T. et al. [77], *Cancer Res* (2005)
Energy metabolisms and respiratory chain dysfunctions	
Hypersensitivity to oxygen	Ishii N. et al. [27], *Mutat Res* (1990)
Decreasing succinate-CoQ oxidoreductase activities	Ishii N. et al. [28], *Nature* (1998)
Increasing superoxide anion production	Senoo-Matsuda N. et al. [29], *J Biol Chem* (2001)
Decreasing mitochondrial membrane potential	Senoo-Matsuda N. et al. [44], *J Biol Chem* (2003)
Lactic acidosis	Ishii T. et al. [77], *Cancer Res* (2005) Tsuda M. et al. [47], *Biochem Biophys Res Commun* (2007) Ishii T. et al. [81], *Mitochondrion* (2011) Onouchi H. et al. [89], *Invest Ophthalmol Vis Sci.* (2012)
Oxidative stress to cellular components	
Increasing lypofuscin, carbonylated proteins and 8-OHdG	Hosokawa H. et al. [42], *Mech. Ageing Dev.* (1994)
Nuclear hypermutability	Adachi H. et al. [43], *J. Gerontol. Ser. A Biol. Sci. Med. Sci.* (1998) Hartman P. et al. [45], *Mech. Ageing Dev.* (2004) I shii T. et al. [77], *Cancer Res* (2005) Tsuda M. et al. [47], *Biochem Biophys Res Commun* (2007) Ishii T. et al. [81], *Mitochondrion* (2011) Onouchi H. et al. [89], *Invest Ophthalmol Vis Sci.* (2012)
Decreasing antioxidant activities	
Decreasing superoxide dismutase (SODs) levels	Senoo-Matsuda N. et al. [29], *J Biol Chem* (2001)
Decreasing glutathione levels	Yasase S. et al. [109], *Mech. Ageing Dev.* (2002)
Alterations cellular and organismal processes	
Premature aging	Ishii N. et al. [27], *Mutat Res* (1990)
Excessive apoptosis	Ishii N. et al. [28], *Nature* (1998)
High-transformation rate	Senoo-Matsuda N. et al. [44], *J Biol Chem* (2003)
Cell cycle arrest	Ishii N. et al. [110], *Mech. Ageing Dev.* (2004)
Accelerated aging	Ishii T. et al. [77], *Cancer Res* (2005) Tsuda M. et al. [47], *Biochem Biophys Res Commun* (2007) Miyazawa M. et al. [80], *BioScience Trends* (2008) Onouchi H. et al. [89], *Invest Ophthalmol Vis Sci.* (2012)

will continue to serve as a valuable whole-animal model for the effects of mitochondrial oxidative stress (Fig. 4).

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