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Invited Mini Review

Mitochondrial superoxide anion ($O_2^{\bullet-}$) inducible "*mev-1*" animal models for aging research

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Most intracellular reactive oxygen species (ROS), especially superoxide anion ($O_2^{\bullet-}$) that is converted from oxygen, are overproduced by excessive electron leakage from the mitochondrial respiratory chain. Intracellular oxidative stress that damages cellular components can contribute to lifestyle-related diseases such as diabetes and arteriosclerosis, and age-related diseases such as cancer and neuronal degenerative diseases. We have previously demonstrated that the excessive mitochondrial $O_2^{\bullet-}$ production caused by SDHC mutations (G71E in *C. elegans*, I71E in *Drosophila and* V69E in mouse) results in premature death in *C. elegans* and *Drosophila*, cancer in mouse embryonic fibroblast cells and infertility in transgenic mice. SDHC is a subunit of mitochondrial complex II. In humans, it has been reported that mutations in SDHB, SDHC or SDHD 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 equilibrates transgene expression to endogenous levels. These mice experienced mitochondrial respiratory chain dysfunction that resulted in $O_2^{\bullet-}$ overproduction. The mitochondrial oxidative stress caused excessive apoptosis leading to low birth weight and growth retardation in the neonatal developmental phase in *Tet-mev-1* mice. Here, we briefly describe the relationships between mitochondrial $O_2^{\bullet-}$ and aging phenomena in *mev-1* animal models. [BMB reports 2011; 44(5): 298-305]

MITOCHONDRIAL REACTIVE OXYGEN SPECIES

Most intracellular reactive oxygen species (ROS), especially superoxide anion ($O_2^{\bullet-}$) that is converted from oxygen, are overproduced by excessive electron leakage from the mitochondrial respiratory chain (1) (Fig. 1). 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) as the final acceptor (2, 3) (Fig. 1). The eukaryotic mitochondrial electron transport system is composed of more than 80 subunits and requires more than 100 additional genes for its assembly (4). 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 ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet$OH) (5) (Fig. 1). 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) (Fig. 1) may constitute as much as 1-2% of total electron flow (6), although others have placed this value at 0.1% (7). It is well known that oxygen is initially converted to $O_2^{\bullet-}$ by electrons leaked from complexes I and mainly complex III (8-11). Complex III generates $O2^{\bullet-}$ by the auto-oxidation of ubisemiquinone (QH_o), formed during the Q cycle (12). $O_2^{\bullet-}$ 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 (13). In contrast, $O_2^{\bullet-}$ generated by complex I is released into the matrix (Fig. 1). 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 (14). In addition, we and another group have demonstrated that $O_2^{\bullet-}$ is produced from complex II in a *C. elegans* genetic background that compromises complex II functionality and from *Ascarissuum* mitochondrial complex II, respectively (15-17). 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 the hypoxic condition (18, 19) (Fig. 2). 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 (20-22) (Fig. 2). These endogenously generated ROS can readily attack a wide variety of intracellular components, resulting in damage that

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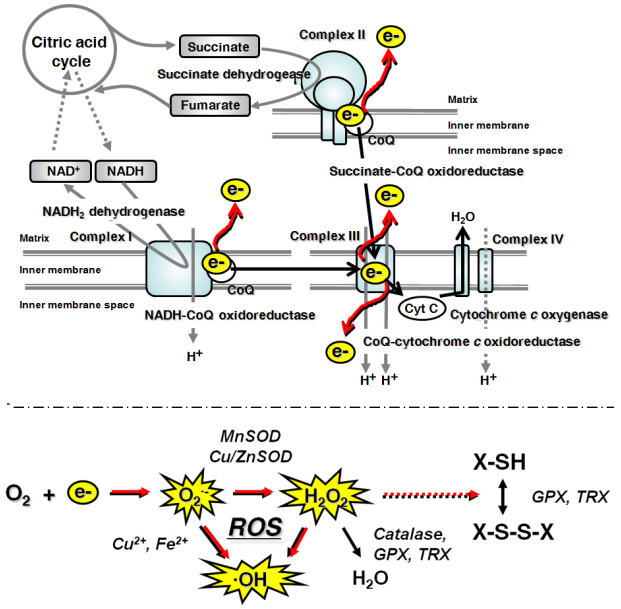

Fig. 1. Mitochondrial electron transport chain and ROS production.

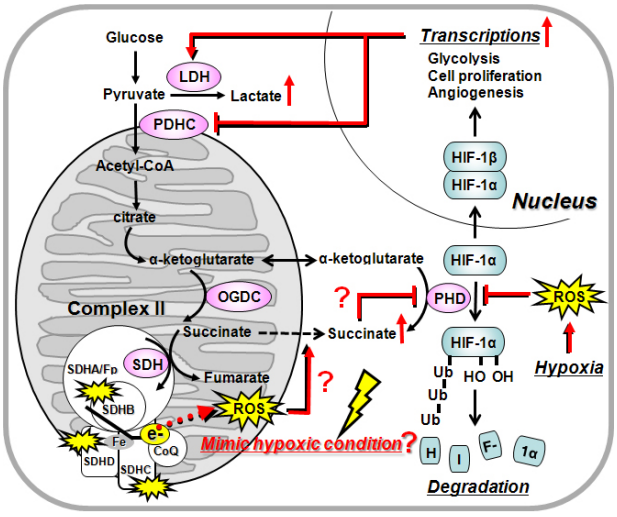

Fig. 2. Mitochondrial complex II dysfunction, ROS production, and energy metabolic change.

compromise cell integrity and function (23-25). This damage can cause or at least contribute to a variety of pathologies, including some in humans (25-30).

C. elegans AND *Drosophila mev-1* MUTANTS WITH SDHC G71E OR I71E 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, and *C. elegans* mitochondrial DNA (mtDNA) is similar in size and gene content to that of humans (31, 32). The *mev-1* (*kn-1*) mutation results in an amino acid substitution at the 71st position from glycine to glutamate (G71E) in *C. elegans* (or I71E in *Drosophila*) and 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 (33, 34). The *mev-1* mutant was isolated based upon its hypersensitivity to the ROS-generating chemical *m*ethyl viologen (35). In addition to its *m*ethyl viologen hypersensitivity, *mev-1* mutants are oxygen hypersensitive with respect to both development and aging. 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 CoQ 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*). *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 (33). 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 CoQ in complex III. Based upon its position, the mutation site in *mev-1* may affect the domain binding to CoQ.

The mean and maximum life spans of both the wild type and *mev-1* mutant were influenced by oxygen (36). Wild-type life spans were not influenced 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 (35). 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 (37-40). 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 (41, 42). 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 (15). 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 (15). The *mev-1* mutation also caused supernumerary embryonic apoptosis especially under hyperoxia (43). 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*. 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* (43). In addition, the oxidative stress by hyperoxia in *mev-1* animals renders them hypermutable to nuclear mutations (44). 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* (15). We have suggested that age-related complex II deterioration might also produce $O_2^{\bullet-}$ and consequently accelerate aging.

HUMAN SDHC MUTATIONS

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 (45-47). The crystal structure of mitochondrial respiratory membrane protein complex II has been elucidated (47). 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. The *SDHD* gene 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 in a genomic region of 38.4 kb. While homozygous germline mutations affecting the *SDHA* gene cause Leigh syndrome, a sub-acute necrotizing encephalomyelopathy during infancy (48-50), *SDHD, SDHB* and *SDHC* heterozygous mutations cause a genetic predisposition to non-chromaffin paragangliomas (PGLs) and adrenal/extra-adrenal phaeochromocytomas (PHEOs) (51-53) 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 602690) as the gene responsible for PGL1 in familial HNPGLs (51), it was subsequently recognized that two other subunits of this mitochondrial enzyme, SDHC (PGL3, OMIM 602413) and SDHB (PGL4, 1p36, OMIM 185470) were associated with heritable PHEO and/or PGL (52, 53). 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, Gimenez-Roqueplo *et al*. (54, 55) studied the biological effects of a loss-of-function *SDHD* germline mutation (p.Arg22X) and a missense *SDHB* germline mutation (p.Arg46Gln) in a malignant PHEO with somatic terminal deletion of 1p. In tumor tissues, SDH activity was abolished with increased expression of HIF-1 alpha, -2 alpha and VEGF in tumor cells associated with increased expression of VEGF-R1 and VEGF-R2 in endothelial cells in agreement with the high vascularization of this endocrine tumor (54, 55). In addition, it has been reported that the inactivation of SDH activity and succinate accumulation was shown to inhibit prolyl-hydroxylation of HIF-1a and HIF-2a, which is an essential step for its degradation through the complex VHL-ElonginD-C-Cul2 (56). We believe that SDH deficiency might trigger hypoxic conditions, resulting in increased activity of HIFs. Collectively, these alterations may trigger changes in cellular metabolism, angiogenesis, cell scattering and cell proliferation (57) (Fig. 2).

MOUSE EMBRYONIC FIBROBLAST CELLS WITH SDHC V69E MUTATION

In order to determine the effects of mitochondrially derived ROS in mammals, we established a transgenic mouse embryonic fibroblast NIH3T3 cell line with the equivalent mutation (V69E) in SDHC as *C. elegans mev-1* (16). The mutation site at the 69th position, changing a neutral amino acid (valine) to an acidic amino acid (glutamate) in SDHC, is located within the functional ubiquinone-binding region of Complex II (45-47). 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. Overexpressed

transgene cell lines were not obtained, most likely because cells with more than 80% abnormal mitochondrial DNA are inviable (58). In addition, RNAi with *cyt-1* produced an embryonic lethal in *C. elegans* (59).

The enzymatic activity of complex II in the *mev-1* cells was reduced to 40% whereas the activity of complex I was unaffected (16). ATP levels were not affected, suggesting that this mutation did not directly compromise cell survival through reduced respiration *per se*.

In the *mev-1* cells, $O_2^{\bullet-}$ production was slightly but not statistically significantly higher in untreated mitochondria. 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 the *mev-1* cells. 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 (16). The *mev-1* cells accumulated cytoplasmic carbonylated 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 (16).

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 (16). During the period of colony formation, some clefts characteristic of cell death were found in the center of some colonies. As expected, the activity of the apoptosis marker caspase 3 was 1.3 to 1.8 times higher in *mev-1* cells (16, 60). 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 (16).

When one-month *mev-1* cells were injected under the epithelium of nude mice, they rapidly disappeared as compared to those of 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 cells resulted in the production of tumors (16, 60). 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 (16). 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 (16). 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 (16, 60). We believe that the *mev-1* like cell lines are suitable models for the tumorigenesis in human such as 'PGL/PHEO syndrome'.

Tet-mev-1 CONDITIONAL TRANSGENIC MICE WITH SDHC V69E MUTATION

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 (61). We employed a modified tetracycline system (Tet-On/Off system) to do so.

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 (62), 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) (63) and the Tc-controlled transcriptional silencer (tTS) with the improved *Kruppel*-associated box (KRAB) domain of human Kox-1 (64, 65) 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 (61). It is also not leaky in the absence of the inducer Dox, which can be problematic at times.

The fourteen lines of *Tet-mev-1* conditional transgenic mice were established using our modified Tet-On/Off construct (61). As was the case with *C. elegans mev-1* and mice embryonic fibroblast *mev-1* cells, these mice were all hypersensitive to methyl viologen, which induces oxidative stress (61). In newborn *Tet-mev-1* mice that had been exposed continuously to Dox from the embryonic stage onward, $O_2^{\bullet-}$ levels were increased compared to those of wild-type mice treated with Dox

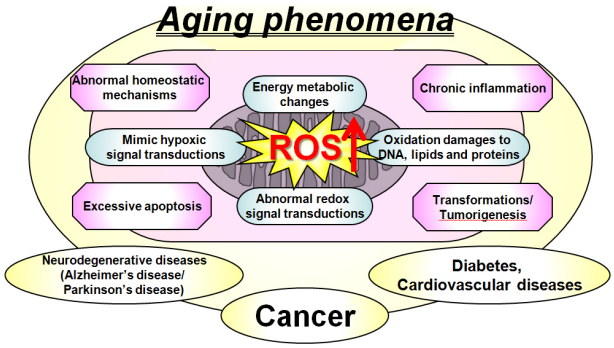

Fig. 3. Mitochondrial ROS, cellular dysfunctions, organismal dysfunctions, and aging phenomena.

(61). 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 C57BL/6j. This included brain, lung, liver, kidney especially in the adrenal region, salivary grand, nasal sinus tissue (especially secretory cells and mucosal cells) and muscles (61). Interestingly, these brown-stained cells 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 oxidative stress that led to excessive apoptosis resulted in a significant decrease in body size and weight and significant developmental and growth retardation in the *Tet-mev-1* mice, just as in the *C. elegans mev-1* mutant (61). In *Tet-mev-1* treated with Dox, the electron transport ratio from complex II to III through ubiquinone (CoQ) 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-CoQ oxidoreductase activity as was the case with malonate-sensitive succinate-cytochrome *c* reductase activity (61).

These phenomena, electron transport ratio, $O_2^{\bullet-}$ production and accumulation levels, excessive apoptotic cell death, low birthweight and growth retardation in *Tet-mev-1* mice were recovered by CoQ supplementation to that of the wild-type C57BL/6j (61). Thus, it was suggested that the electron leakage that led to $O_2^{\bullet-}$ overproduction from mitochondria resulting in the low birthweight and growth retardation during the neonatal period with excessive apoptosis was caused by a decrease in the affinity of CoQ for complex II with mutated SDHC V69E.

In addition, we have recently analyzed a variety of additional *Tet-mev-1* mouse phenotypes. The data would suggest that these animals will continue to serve as a valuable whole-animal model for the effects of oxidative stress (Fig. 3).

CONCLUSIONS AND FUTURE DIRECTIONS

Chronic elevation in ROS levels presumably result 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 (Fig. 3).

Oberley and colleagues argued the importance of $O_2^{\bullet-}$ in cancer, differentiation as well as aging (66-68). 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 carcinogenesis (Fig. 3).

A mutation in *SDHB, SDHC* or *SDHD* was found in patients of PGL/PHEO syndromes (51-55). 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, cell scattering and cell proliferation that could result in tumorigenesis (56, 57, 69) (Fig. 2). In addition, it has been well known that oxidative stress with mitochondrial dysfunctions can result in excessive apoptosis or chronic inflammation. These can contribute to neurodegenerative diseases, cardiovascular diseases and diabetes (29, 30) (Fig. 3).

In conclusion, ROS production from mitochondria may lead to pathologies such as precocious aging, neuronal degeneration, cardiovascular diseases, diabetes and tumorigenesis in humans. Thus, we anticipate that the *mev-1* models will be suitable for not only for the study of tumorigenesis and carcinogenesis such as PGL/PHEO syndromes, but also some age-related disease models; for example, 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. 3).

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