Oxidative Modification of SOD1 As a Mechanism of Pathological Aging

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Abstract

Aging-associated stress induces post-translational oxidation at cysteine 111 residue in both mutant and wild type of SOD1, which is one of our primary antioxidant enzymes. Such oxidatively modified SOD1 influences the oxidation of other native proteins in its vicinity and becomes misfolded and forms aggregates that propagate in a prion-like manner from cell to cell in age-related diseases like ALS, AD- and PD. Assuming the cumulative consequence, we have hypothesized that oxidatively modified SOD1 is a mechanism of pathological aging. Both in vivo and in vitro, we have utilized the mutant human SOD1 (G93A) as a tool to prove this hypothesis as they are the most commonly studied model for ALS research and more vulnerable to oxidative stress than the wild varieties. Hence, pathological consequences are more conspicuous and understandable. Western blot analyses and immunofluorescence studies were performed in N2a cells, transiently transfected with G93A plasmid constructs, which revealed robust senescence compared with the cells transfected with wild type SOD1. In addition, MalPEG modification and subsequent WB analysis of the spinal cord tissues collected from the different age groups of G93A transgenic mice showed that oxidative modification of human SOD1 increased in an age-dependent manner that coincided with the rise of cellular senescence. Then, we selectively knocked down this modified human SOD1 by transfecting the N2a cells with CT4 plasmid construct that were initially transfected with G93A and by injecting CT4 fusion peptide intraperitoneally for one month to the G37R transgenic mice. The binding efficiency of CT4 peptide to modified human SOD1 was confirmed by GST pull-down assay. MalPEG modification and western blot analysis demonstrated a significant decrease of oxidatively modified human SOD1 and cellular senescence in both experimental models after implicating the peptide treatment. The findings are in agreement with our hypothesis; oxidized SOD1 is a mechanism of cellular aging in ALS pathogenesis. Besides, CT4 mediated knockdown of modified SOD1 could be an effective strategy to prevent cellular aging and early death.

Abbreviations

AAV adeno-associated virus

ALS amyotrophic lateral sclerosis

AD Alzheimer's disease

AIF apoptosis-inducing factor

ALP autophagy-lysosome pathway

ATG autophagy-related genes

ATF6 activating transcription factor 6

Bad tBcl-2-associated death

Bax Bcl-2-associated X protein

BBB blood-brain barrier

Bcl B-cell lymphoma

BDNF brain derived neurotrophic factor

bFGF fibroblast growth factor

C9ORF72 chromosome-9 open reading frame

CACS central animal care services

CHO Chinese Hamster Ovary cells

CMA chaperone-mediated autophagy

CNS central nervous system

CSF cerebrospinal fluid

CT-1 cardiotrophin-1

CTM chaperone-mediated autophagy-targeting motif

DAPK1 death associated protein kinase 1

DBR Derlin-1 binding region

Dubs deubiquitinases

EAAT excitatory amino acid transporter

EAE experimental autoimmune encephalomyelitis

EBSS Earle's balanced salt solution

EM electron microscopy

ER endoplasmic reticulum

ERAD endoplasmic-reticulum-associated degradation

fALS familial ALS

FUS fused in sarcoma

GFAP glial fibrillary acidic protein

GLTs glutamate transporters

GLUT glucose transporters

GRP glucose-related protein

H_2O_2 hydrogen peroxide

hEGF human epidermal growth factor

HSP heat shock proteins

ICC immunocytochemistry

IP Intraperitoneal injection

iPSCs human induced pluripotent stem cells

IRE1 inositol-requiring enzyme 1

LAMP2 lysosomal associated membrane protein 2

LMN Lower motor neuron

MAPK mitogen-activated protein kinase

MCT monocarboxylate transporter

MeCP2 methyl CpG binding protein 2

MRI magnetic resonance imaging

MS multiple sclerosis

mSOD1 mutant SOD1

NFκB nuclear factor κB

NGF nerve growth factor

NMDA non-N-methly-D-aspartate

NMJ neuromuscular junction

NRP1 neuropilin 1

NSC neuronal stem cells

NTFs neurotrophic factors

OPC oligodendrocyte precursor cell

PBS phosphate-buffered saline

PD Parkinson's disease

PDL poly-D-lysine

PDI protein disulphide isomerase

PEG polyethyleneglycol

PERK protein kinase R-like ER kinase

PFA paraformaldehyde

PLA proximity ligation assay

PNS peripheral nervous system

POIs protein of interest

PPX Pramipexole

PQC protein quality control

qRT- PCR quantitative reverse transcriptase polymerase chain reaction

ROS reactive oxygen species

RPPX dexpramipexole

RRMS relapsing remitting multiple sclerosis

sALS sporadic ALS

SC spinal cord

Sema semaphorin

SOD1 superoxide dismutase 1

TARDBP TAR DNA-binding protein

TNFα tumor necrosis factor alpha

Acknowledgment

Coming from a clinical background, I was going through a crucial metamorphosis throughout my master's research in molecular neuroscience. The journey was nerve-wracking, with several attempts to relinquish during my initial months. In retrospect, I realized that behind the revolutionary hypothesis and rigorous bench work, the ultimate way of being young forever is to explore the unfamiliar territories every now and then.

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Chapter 1 Introduction

1.1. Aging

Generally, aging is described as a perplexed, multifactorial, and time-dependent process that gradually declines physiological integrity and physical capabilities. So far, no single theory has comprehended aging conclusively, yet recently, two of them: the programmed aging theory and the damage or error-based theory are prevailing. The first one suggests that the structural and functional deterioration associated with aging is intrinsically programmed, whereas the later one claims the external and internal damages cumulatively leads to aging. More recent times, the amalgamation of these theories have established nine potential hallmarks of biological aging including (1) genomic instability, (2) telomere attrition, (3) epigenetic alterations, (4) loss of proteostasis, (5) deregulated nutrient sensing, (6) mitochondrial dysfunction, (7) cellular senescence, (8) stem cell exhaustion, and (9) altered intercellular communication. Comprehensively, biological aging can be defined as molecular, cellular, and systemic deterioration leading to compromised adaptability to stress, homeostatic imbalance, and increased risk of developing age-related diseases.

1.2. Aging Classification

Chronological aging*:* chronological aging is the time spent since birth, which is often utilized as a control to compare other forms of aging. It is continuously progressive and unbiased by external influences (1).

Psychological aging: Psychological aging is a qualitative assessment of how old one feels, acts, and behaves, and is not necessarily equal to chronological age. Successful psychological aging demands an age-friendly environment and support system involving family and friends(2).

Social aging*:* It is a numerical scale unit that refers to the maturity level of a person in terms of his or her interpersonal skills and ability to meet the norms and expectations associated with particular social

roles and usually compared with control of the same chronological age. It is analogous to mental age and assessed by the ratings gathered from the relevant people, such as in case of a child, it could be the parents or the caregivers using instruments like ‘Vineland Adaptive Behavior Scales’ (2).

***Biological agi*ng**: The biomolecular research on aging or gerontology and the aging process examined in the current study revolve around biological aging. Whether biological aging is a disease or a physiological entity, has dispersed conflicting arguments from ancient times. The hallmarks of biological aging mentioned in section 1.1 are evident during healthy aging. However, experimental aggravation or amelioration of these factors accelerate or retard the aging process, respectively. Such findings have yielded two phenotypes of biological aging: healthy aging and pathological aging, which are triggered by common molecular and cellular mechanisms and are responsible for the heterogenicity in the aging population. Pathological aging similar to genetic disorders such as Down Syndrome, Hutchinson-Gilford Progeria, Werner’s Syndrome manifests as accelerated aging. In some instances, like Parkinson’s disease, it is indistinguishable from healthy aging due to the long subclinical incubation period. Consequentially, the complex interaction of genetics and environment, efficiency of the anti-oxidant system, immune system, and the signal transduction pathways related to aging, eventually determine the phenotype between healthy and pathological aging.

1.3. Biomarkers of Aging

Biomarkers of aging are defined as biological parameters that can distinguish age-related changes in body function or composition and anticipate the commencement of age-related diseases and a residual lifetime more accurately than the chronological age (3). An established biomarker must meet the following criteria (4)

- progressively changes with age
- predicts mortality better than the chronological age
- foresees the early stages of age-related diseases
- can be assessed repeatedly, utilizing accessible samples by minimally invasive harmless procedures in both laboratory animals and humans.

1.3.1. Molecular Biomarkers Associated with Aging

Biomarkers are classified based on the hallmarks of aging, or according to the central dogma of molecular biology, they are also described as DNA, RNA, and protein-associated biomarkers.

1.3.1.1. DNA Associated Biomarkers

Leukocyte telomere length is a conventional marker and commonly assessed by qPCR or TRF (Terminal Restriction Fragment) analysis, which is the gold standard technique (5). The typical oxidative lesion, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), is frequently detected as a DNA damage marker. However, γH2AX, a phosphorylated variant of histone H2A family, constitutes an initial response to DSBs and is a regularly analyzed biomarker of DNA damage. It is a persistent biomarker that accumulates in genomic, telomeric DNA, and senescent cells and carries a prognostic value to assess the repair and recovery from the initial injury(6). Recently, cell-free DNA (cfDNA) in the circulation is sequenced, which is a non-invasive technique to determine the alteration in the genetic landscape throughout the aging process. These are released from the apoptotic cells, and cfDNA loss is higher at selective genomic locations such as the site of transcription commencement and termination, retrotransposons, indicating the vulnerability of these regions during aging (5).

DNA damage-induced senescence is independent of epigenetic aging. The epigenetic clock refers to the intrinsic mechanism of DNA methylation that exists from birth and could predict biological age with 3.5

years of accuracy(7). Methylation profiling in human blood samples indicated that CpG sites located at gene ITGA2B, ASPA, and PDE4C were strictly correlated with aging. Epigenetic aging also involves post-translational modification of histones and chromatin remodeling (8).

1.3.1.2. RNA Associated Biomarkers of Aging

The evolution of post-genomic tools has established a new perspective in the selection of aging biomarkers. Transcriptomic age is measured in blood samples utilizing specific RNA fractions.

Circulating microRNAs (miRNAs) are classes of small non-coding RNAs consisting of 21 to 23 nucleotides. They are easy to access and stable biomarkers in plasma that reside in exosomes or bind to protein and lipoprotein factors (9). Some of the potential miRNAs analyzed as biomarkers in the context of healthy aging and pathological aging are listed in Table 1.

Another transcriptome fractions, known as long non-coding RNAs (lncRNAs), consists of transcripts longer than 200 nucleotides. They regulate the gene expression pattern in ARDs, modulates energy metabolism, immune response, neurodegeneration, and proliferative processes. Their knockdown is found to be associated with dysregulated autophagy response and cell cycle pathways leading to cellular senescence (10).

1.3.1.3. Metabolic Biomarkers of Aging

The metabolic biomarkers of aging include 1) markers of systemic metabolism such as blood urea nitrogen(BUN) and creatinine, metal-binding proteins (transferrin, α_2 macroglobulin) fasting glucose, glycosylated Hb (HbA_{1c}), basic parameters like albumin, serum protein concentration 2)products of fatty acid, and cholesterol metabolism, for instance, fasting triglycerides, free fatty acids, total cholesterol, HDL and LDL cholesterol, lipoprotein particle size and 3)markers of systemic inflammation like C-reactive protein (CRP), homocysteine, fibrinogen, serum amyloid A and P, vitamin D,

Dehydroepiandrosterone sulfate (DHEAS). In addition, oxidative damage induces metabolic stress. Prospective biomarkers have been considered, which are modified bi-products of metabolic reactions such as malondialdehyde, carbonylated and nitrated proteins, oxidized LDL, NO-metabolic pathway products, cellular glutathione (11)(3).

1.3.1.4. Cellular Biomarkers

Cellular biomarkers include the markers of cellular senescence, mitochondrial dysfunction, and intercellular communication. It would be perplexing to rely on a single marker to examine the cellular senescence. Instead, senescence is validated by evaluating a series of markers like senescence-associated β-galactosidase activity (SA-β-Gal)(12)(13), based on the stable growth arrest, markers of proliferation like Ki-67 and 5-bromodeoxyuridine (BrdU) are regularly assessed tools. Additionally, p53/p21 and p16INK4a^{INK4a} proteins are upregulated during cellular senescence and are quantified to determine the cell cycle arrest (14)(15). Besides, senescence-associated heterochromatic foci (SAHF), senescence-associated secretory phenotypes (SASPs) such as pro-inflammatory cytokines and chemokines (e.g., the interleukins IL-6, IL-1, and IL-8), growth factors (IGF-binding proteins, their regulators and transforming growth-factors) are frequently evaluated senescence markers(16).

Age-associated decrease in lon protease activity, altered aconitase turnover, decelerating membrane potential, reduction in mitochondrial enzymes like citrate synthase, reduced mitochondrial biogenesis indicated by PGC1α, diminished ATP production are some of the biological parameters of aging conferred from damaged mitochondria(17).

SASPs may act in an autocrine and paracrine manner with a high level of interleukin-6, tumor necrosis factor-alpha, monocyte chemoattractant protein-1, matrix metalloproteinases, and IGF binding proteins in the circulation. Besides, damage-associated molecular pattern molecules (DAMPs) such as heat shock proteins, histones, high-mobility group box 1, and S100, compose a group of molecules released after

injury or cellular death and mediate the immune response. They can contribute as stress inducers and mediators by interacting with the individual plasma membrane receptors, intracellular recognition receptors (e.g., advanced glycosylation end product-specific receptors, AIM2-like receptors, RIG-I-like receptors, and NOD1-like receptors, and toll-like receptors), or following endocytic uptake. DAMPs represent ideal biomarkers of aging and provide an attractive target for interventions in aging and age-associated diseases(18)(19).

Table 1: Biomarkers of aging

Biomarker class	Biomarker Subclasses	Biomarkers	Detection techniques
DNA associated biomarkers	Telomere	Leukocyte telomere length	TRF*, qPCR
	DNA damage & repair	8-oxo-dG, γH2AX	HPLC with tandem mass spectrometry, qPCR, Immunohistochemistry
	Epigenetic modification	DNA methylation	HPLC-UV, ELISA, PCR, LC-MS/MS
RNA associated biomarkers	Circulating microRNAs (miRNAs)	miR-34a, miR-21, miR-126-3p miR-151a-3p, miR-181a-5p, miR-1248	Single cell RNA sequencing
	Long non-coding RNAs	MIR31HG, AK156230, Meg3	
Metabolic biomarkers	Systemic metabolism & toxicity parameters	BUN, creatinine, HbA_{1c}	Colorimetric assay, HPLC, OGTT, Mass spectrometry (MS), ELISA
	Fatty acid and cholesterol metabolism parameters	Triglyceride, cholesterol, HDL,LDL	
	Systemic inflammation parameters	CRP, homocysteine, fibrinogen, DHEAS	
	Oxidized metabolic products	Malondialdehyde, 3-nitrotyrosine, 3-chlorotyrosine, o-tyrosine	
Cellular markers	Senescence Markers	P53, p16, β-Gal, SASPs	WB, qPCR, β-Gal staining, ELISA
	Mitochondrial Dysfunction	Membrane potential, ATP production, enzymatic activity	Seahorse*, Mito tracker probes, Flowcytometry, Assay kit, Immunofluorescence study, WB,ELISA
	Intercellular communication	IL-6, TNFα, metalloproteinase, DAMPs	

TRF- telomere restriction fragment analysis, HPLC- High performance liquid chromatography, LC-MS/MS- Liquid Chromatography with tandem mass spectrometry, ELISA-enzyme-linked immunosorbent assay, DAMPs- damage associated molecular pattern. * Gold standard

1.4. Molecular mechanism of Aging

1.4.1. *Telomeric and non-telomeric DNA damage:*

Oxidative stress amplifies the telomere shortening by 3-20 fold compared to the regular loss of base-pair per replicative cycle (20). Non-telomeric DNA damages include single or double strands break, intra-strand or inter strands cross-linking, oxidation of the DNA bases, especially guanine. The typical oxidative lesion, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG), is detected at a higher level in mtDNA (mitochondrial DNA) than the nuclear DNA, suggesting the susceptibility of mitochondrial DNA to oxidative damages. Three plausible mechanisms might be: a) its proximity to the source of ROS (Electron Transport Chain); b) it lacks "protective" histones, and c) a limited repertoire of DNA repair pathways available in mitochondria (21).

1.4.2. *Age-related epigenetic modification:*

Epigenetic modifications relevant to aging comprehends: a) global alleviation of DNA methylation; b) multiple methylations of CpG sites in CpG islands leading to silencing of regulatory genes controlling transcription, apoptosis, development, and differentiation; c) alterations in DNA methylation enzymes; d) histone modification affecting chromatin compaction; and e) expression of age-related genes controlled by noncoding RNAs (ncRNAs), both short ncRNAs (mostly microRNAs) and long ncRNAs (22)(23).

1.4.3. Oxidative modification of proteins and potential consequences

PTM is a process by which the freshly minted polypeptide chains, propagated from the protein building apparatus, get altered by enzymatic or non-enzymatic attachment of chemical groups at their peptide backbone and amino acid side chains (24). Due to abundance and enormous functional involvement, proteins are the most vulnerable targets of oxidative stress-induced post-translational modification. A vast majority of age-related diseases such as cataract (25), arthritis (26), cardiovascular diseases (CVD),

chronic kidney disorders (CKD)(27), cancers (28) amyotrophic lateral sclerosis (ALS) (29) (30), Huntington's disease (HD) (31) are associated with single or multiple abnormal PTMs. There are different forms of oxidative modifications, including oxidation, carbonylation, glutathionylation, and cysteinylation. Among these modifications, oxidation is the most elaborately studied post-translational protein modification. The reaction either targets the protein backbone causing fragmentation or interacts with the amino acid side chains, which extends toxic conformational changes in the protein. The later confers more complex modifications such as misfolding, aggregation with several pathological fallouts (28).

1.4.4. *Molecular dysregulation of signal transduction pathways during aging*

Numerous proteins within the mTOR signaling pathway network can become dysregulated; for example, the homeostasis between AKT stimulation and PTEN inhibition could be altered to a variable extent during aging (32). The highly active *Insulin/Insulin-like growth factor* pathway generates ROS, inhibits autophagy, and stress response that results in diminished cell resilience and accumulation of cellular debris(33). FOXO is down-regulated by aging-associated oxidative stress. At low oxidative stress, FOXO is upregulated to strengthen the antioxidant defense, reduce DNA damage and revert the mitochondria to its standard size (FOXO3A), establishing itself as one of the critical modulators of aging (34). TGF-β deprives the old cartilage of its protective effects by adopting another signaling via ALK1 signaling (Smad1/5/8 route) instead of ALK5 (Smad2/3 route), which is the basis of aging-associated osteoporosis. Chronic low-grade inflammation induced by NF-κB could probably contribute to aging (35). Besides, aging-related hormonal changes and interleukin secretion could significantly alter the aging process. Such as IL-1 is associated with cartilage destruction, estrogen deficiency after menopause negatively regulates bone remodeling (36).

1.4.5. Aging-associated alterations in proteostasis

The concept of proteostasis implies a comprehensive biological pathway inside the cells that regulate the biogenesis, native folding, trafficking, and degradation of the proteins within and outside the cells (37,38). During aging, peroxiredoxins like Tsa1 recruit chaperons HSP70 and HSP104 to disaggregate the misfolded proteins (39). Aging-associated reduction in ATP synthesis simultaneously reduces the ATP dependent chaperons. Chronic stress leads to the formation of metastable proteins that act as a sink to trap the chaperons. Again, aging interrupts the chaperoning activity by unrecognizable post-translational modifications in protein, such as advanced glycation products(37). The proteins required for endosomal trafficking, intracellular vesicle, autophagolysosome formation are reduced with age (40). However, protein degradation machinery has a dominant role in maintaining the proteostasis by eliminating the aberrant proteins, which comprises ubiquitin-proteasome and lysosome-autophagy mediated pathways.

With aging, the system loses its efficiency by a reduction in protein ubiquitination, subunit expression (Rpn6, α2-α5, β1-β5) and the proteasome assembly (41).

Autophagy-lysosomal pathway

It includes macroautophagy, microautophagy and chaperon mediated autophagy. ALP mostly recognizes and removes the vesicle-mediated large protein complex, aggregates, and dysfunctional organelles. The insoluble misfolded aggregates that are features of pathological aging in the brain are supposed to be removed by this system. Pathological aging is marked by a general reduction in autophagy induction by suppressed mTOR signaling, global defect in cargo recognition by autophagosome, phagophore formation, and depleted autophagosome-lysosome fusion.

Chaperon Mediated Autophagy (CMA)

Approximately 30% of the cytosolic proteins are degraded by this process(42). LAMP-2A and HSC70 are two substantive players of CMA. While LAMP-2A works as a receptor for protein substrates at the lysosomal membrane, HSC70 specifically binds protein targets and takes them for CMA degradation. Because of the broad spectrum of proteins able to be degraded by CMA, this pathway is involved in physiological and pathological processes such as lipid and carbohydrate metabolism, and neurodegenerative diseases, respectively (42). It varies from macroautophagy in three aspects: (i) needlessness of autophagosomes and autolysosome formation, (ii) instead of organelles; it targets cellular proteins; and (iii) the protein cargo interacts with HSC70 and LAMP-2A and is directly delivered into the lumen of the lysosome. Both HSC 70 and LAMP-2a are reduced in expression during the aging process(43).

1.5. Aging in organelles

It refers to the structural and functional changes that substantially contribute to the deterioration of the tissue function during aging. Organelle aging is a regulatory hub to cellular aging that is more conspicuous and perilous in mitochondria and endoplasmic reticulum (44).

1.5.1. Mitochondria

Healthy aging in mitochondria is conferred by an intricate balance between mitochondrial fission (Drp1, Mfn, Opa1) and fusion (Mfn, Drp1) cycle. It is speculated that during healthy aging, tissues with a high metabolic rate like muscle, brain, liver mostly rely on mitophagy to remove dysfunctional organelles, are expected to show a high level of fission markers compared to the peripheral organs with low metabolic rate. Enlarged tubular mitochondria characterize increased fusion with an accumulation of damaged mitochondria, oxidative stress, and less biogenesis. On the other hand, abnormal fission leads

to increased mitophagy with similar consequences by fostering a fragile mitochondrial DNA integrity (45)(46).

In the inner mitochondrial membrane, disorganized and swirled cristae evoke respiratory enzyme COX deficiency. Eventually, loss of crista leads to dissociation of mitochondrial ATP synthase dimer and disrupt the resistance against membrane elastic energy. Subsequently, altered oxygen uptake, free radical production becomes evident with damaged and deformed mitochondria. Overall, metabolic activity in ETC, membrane potential, and Ca homeostasis are mitigated (47).

1.5.1.1. MQC (Mitochondrial Quality Control)

Mitochondria adopt four alternate pathways in response to stress (48). Proteases in the mitochondrial matrix and inter-membrane space act as a first-line defense against unfolded and oxidized soluble proteins. Outer membrane proteins are instead removed from the mitochondria through a retrotranslocation pathway following ubiquitination. Degradation of these proteins is completed within the cytosolic proteasome, similar to the ER-associated degradation pathway. Besides, in response to mild damages, un-depolarized mitochondria manufacture the mitochondrial-derived vesicles (MDVs) that travel to lysosome or peroxisomes (10%-20%) for degradation (49). PINK1-Parkin mediated MDVs are double or single membrane vesicles that are assumed to be either Tom20 or PDH positive. This shuttle system is versatile as it does not require mitochondrial depolarization, autophagy signaling, or mitochondrial fission. The formation of MDVs is an early response to oxidative stress in MQC. In contrast, severely damaged mitochondria releasing oxidants and pro-apoptotic factors are fissioned and destined for elimination by mitophagy. Only upon complete mitochondrial dysfunction, or failure of most of the import channels, the entire organelle would be targeted to the autophagosome (46).

1.5.2. UPR (Unfolded Protein Response) and ER-phagy

ER stress is characterized by dynamic protein synthesis, the imbalanced $Ca2^{+}$ homeostasis, and the inhibited protein modification or interrupted transfer of proteins to the Golgi body(50). UPR targets to restore the ER function and mitigate ER stress. Besides, the UPR also eliminates abnormal cytotoxic proteins across the ER membrane by an efficient retrotranslocation system from ER lumen and membrane to the cytosol for degradation through EARD (endoplasmic reticulum-associated degradation), which involves ubiquitination and proteasome-mediated degradation (51). However, if ER homeostasis or function cannot be re-established, programmed cell death will be activated by the UPR, presumably to safeguard the entire organism from the cells that display misfolded proteins (52).

ER-phagy, similar to mitophagy, is a proteostasis mechanism featuring autophagic degradation of ER. In macro-ER-phagy, autophagosomes enclose fragments of the ER and fuse with lysosomes to degrade the internal material containing the ER, whereas, in micro-ER-phagy, invaginated membranes of lysosome 'pinch off' parts of the ER into the lysosomal lumen. Also, lysosomes can directly fuse with ER-derived vesicles for degradation (53).

1.6. Cellular Aging: Senescence

The concept of senescence is often intermingled with aging and utilized as a contemporary term to replace aging. In fact, aging and senescence are two different phenomena where senescence is a downstream consequence of molecular and organelle aging and an upstream influence on cell death (54).

Senescence originated from the Latin word 'senex' means 'growing old,' is generally defined by the state of irreversible cell cycle arrest induced by short and uncapped telomeres or by stress-induced molecular alteration and organelle dysfunction (55).

Telomere shortening with consecutive cell cycles, implemented in replicative senescence, is more relevant to the normal/ healthy aging. In contrast, stress-induced senescence is relatable to pathological aging. It is accelerated cellular senescence instigated by various stimuli such as aging-associated oxidative stress, aberrant oncogene (RAS and RAF) activation (oncogene-induced senescence-OIS), ionizing radiation, DNA-damaging chemotherapy, hyperoxia, impaired autophagy, mitochondrial dysfunction and ER stress and other stressors (56). Senescence is a protective mechanism that halts the progression of damaged cells for the time being and activates the repair mechanism. On the other hand, persistent senescence resulting from a saturated repair mechanism leads to deleterious consequences by 1) secreting SASP that acts in an autocrine and paracrine manner to induce further the oxidative stress and a state of chronic inflammation 2) interrupting stem cell proliferation to replace the damaged cells 3) losing physiological activity of the senescent cells (45).

1.6.1. Morphological and Molecular Features of Senescence:

Morphological characteristics of senescence include flattened enlarged cells with increased beta-galactosidase staining, elongated nucleus, and nucleoli along with multinucleated cells and increased number of lysosomes, Golgi apparatus, and cytoplasmic microfilaments(57). Pleomorphic giant, highly interconnected mitochondria are formed during cellular senescence because fusion is a complementary approach to maintain homogenous inheritance to progeny, to transfer energy efficiently, and to preserve the function of the randomly damaged individual mitochondrion(45).

At the molecular level, the earliest feature of senescence is upregulation of p53 and downstream activation of CDKi (cyclin-dependent kinase inhibitor) p21 at the G1-S interface. $P16^{INK4A}$ mediates persistent cell cycle arrest by inhibiting CDK4 and CDK6, which leads to RB (retinoblastoma) hypophosphorylation and blocks entry to the S phase. Apart from these, senescence-associated

secretory phenotypes (SASP), senescence-associated beta-galactosidase (SA-β-Gal), and chronic inflammatory cytokines are the essential markers of senescence (58).

1.6.2. Senescence in Post Mitotic Cells

Post mitotic cells such as neurons, cardiac myocytes, skeletal muscle cells reside in the G° phase of the cell cycle, which is a permanently non-dividing, metabolically active, functional state. Senescence in post-mitotic cells was initially argued because of their non-proliferative existence in the cell cycle. The earliest concept is challenged by the detection of conventional senescence markers mentioned above in the non-diving cells like brain cells, including neurons (59). It is hypothesized that a quiescence like stress response could exist in mature neurons. It is known as 'amitosenescence,' which could further transit from the quiescent state to alert form. In response to stress-induced DNA damage, neurons in the G0 phase may adopt an unscheduled cell cycle activity in an attempt to reinitiate the S phase. The analogy is quite similar to the quiescent stem cells that re-enter the cell cycle to proliferate.

Nevertheless, the clumsy replication attempts in neurons are identified by the DNA Damage Response (DDR), which instigates the downstream production of proteins associated with proliferative arrest such as p53, p16INK4A. Eventually, other features of senescence become evident in the post-mitotic cells shown in figure 1.1. Currently, stress-induced aberrant cell cycle progression and subsequent senescence in post-mitotic cells are termed as 'Psudomitosenescence'(60).

In contrast, senescence in post-mitotic cells might simply indicate their wear off in functionality and progression towards cell death. Therefore, qualitative assessment of neuronal motor activity (i.e., muscle response) or analyzing the cognitive impairment and measuring the viability could be a more relevant index of aging in postmitotic cells.

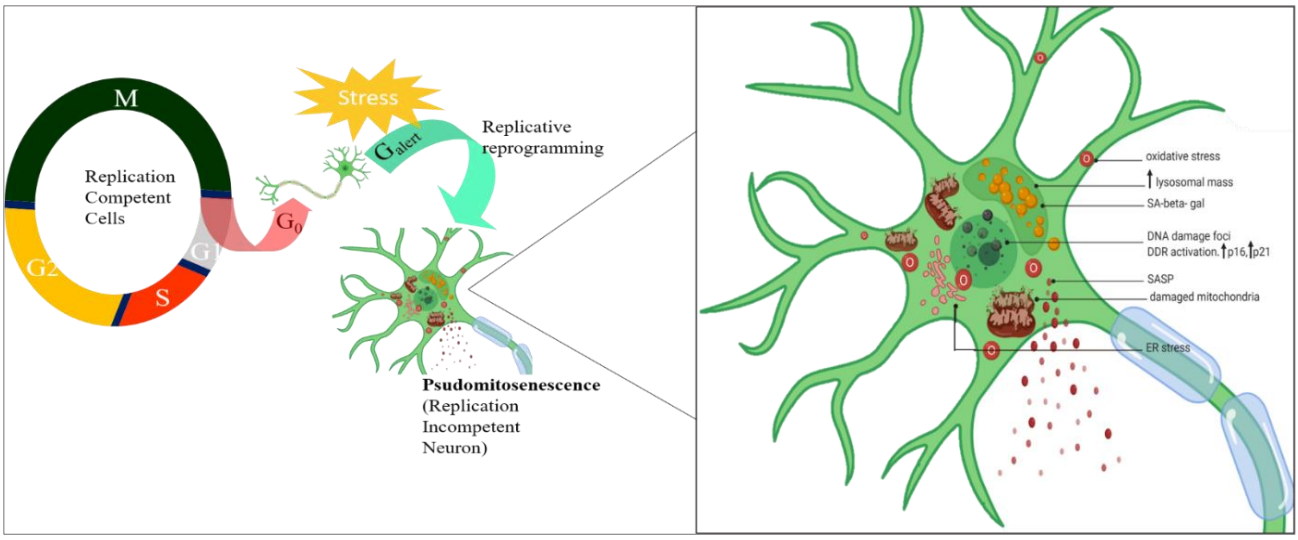

Figure 1: Pseudosenescence in neurons.

The neurons in G_0 phase may enter into a G_{alert} phase by stress stimulus to re-enter into the cell cycle and ends up in a senescence-like state (left). The expression of neuronal senescence by typical features is shown in the right image.

1.7. Amyotrophic Lateral Sclerosis (ALS): An Ideal Example of pathological aging

ALS is a severely progressive and globally lethal age-related disease with underlying degeneration of upper and lower motor neurons (UMN & LMN) and concomitant glial pathology. 90% of the ALS cases are sporadic while remaining 10% are familial ALS linked to mutations in various genes, mostly, chromosome-9 open reading frame 72 (*C9ORF72*), SOD1*(Superoxide dismutase 1)*, TAR DNA-binding protein (*TARDBP*), fused in sarcoma (*FUS*)(70).

The symptoms of UMN dysfunction include spasticity, hyperreflexia, wasted limbs, and the Babinski sign. Patients with UMN-dominant ALS usually show significant younger disease onset than that in classic ALS patients, as well as different-sex ratio, spreading pattern, and clinical phenotype(71).

The symptoms of LMN dysfunction include fasciculation, muscle cramps, and muscle atrophy. Although LMN signs are predominant in the majority of ALS cases, a combination of UMN and LMN signs is still a significant contributor to phenotypical heterogeneous presentations of ALS cases.

Notably, although ALS has been generally considered as a motor neuron disease, the pathological alternations are not limited to motor neurons only(71). The study revealed that the frequency of cognitive impairment in patients with ALS is higher than that in patients with other neuromuscular diseases. Besides, those ALS patients with cognitive impairment had significantly shorter survival time compared to those without cognitive symptoms.

1.7.1. lifespan in ALS

According to a U.S.A based study, published and revised by ALS Association in May 2019, 15 new cases of ALS are detected per day with more than 5,000 cases per year (http://www.alsa.org/about-als/facts-you-should-know.html). The estimated cases of ALS in Canada are currently 3000, and 2-3 of them die every day. Every 90 minutes, there is a new case of ALS with one patient dying without a cure. The peak age of disease onset is 58 to 63 for sporadic form and 47 to 52 for the familial variety. The average life expectancy with ALS is only 2-5 years after the disease onset(71). The disease progression in ALS is fatal, involving neurons and glial cells, multiple organelles especially, ER and mitochondria, and initiates a cascade of the inflammatory responses in the internal milieu (72,73) A multifaceted disease pathology associated with ALS jeopardize the therapeutic interventions due to which it is challenging to halt the disease progression. Instead, delaying the pathological aging in ALS by targeting the disease onset is a rational approach to increase the healthy life span in these patients.

1.8. Superoxide Dismutase 1 (SOD1) and its association with ALS

Since the functional characterization of SOD1 in 1969, the dismutation reaction against superoxide free radical is elaborately analyzed (74). Besides, the functional SOD1 encounters additional PTMs (31); for example, in response to oxidative DNA damage, phosphorylation of the serine residues allows the nuclear translocation of SOD1 where it induces the transcription of several antioxidant genes by binding

to specific promoter regions (75). Also, SOD1 has shown its association with longevity-promoting factors such as SIRT5 that controls mitochondrial respiration by maintaining SOD1 in a desuccinylated state at Lysine 122. Thus, it enables SOD1 to inhibit complex I of the electron transport chain (ETC) to suppress mitochondrial respiration (76).

Apart from these extended physiological stress responses, 25% of familial ALS (fALS) are associated with genetic mutations in SOD1 (77), whereas 5% of the sporadic ALS (sALS) have shown alliance with the wild variety of SOD1. Transgenic mice, overexpressing mutant forms of hSOD1, mimic the phenotypic trajectory of the human condition in ALS (78). Studies with the G93A mouse model have shown that the onset of the disease is relative to the copy number of SOD1 transgene. For instance, G93A mice expressing 25, 18, 13, and 10 copies of human SOD1 have exhibited disease onset at around 90, 90-120, 200, and 300 days accordingly, which reflect a negative correlation between mutant SOD1 and the onset of pathological aging in fALS (79). However, increasing evidence indicates that the toxic gain of function in SOD1 is the disease-causing mechanism (80) in both fALS and sALS, where genetic predisposition could be considered as an aggravating factor that accelerates the pathological aging further. It is speculated that both mutant or wild variety of SOD1 forms post-translationally modified non-native conformations that are adamant to breakdown by internal degradation machinery and exert their toxic effects like a cobweb manner over the native molecules and the functioning apparatus of the cells (80).

Table 2: ***Progressive phenotypic correlates in the G93A murine model of ALS1 Age*** (81)(82)(83)(84)

Genetic modification	# of gene copies	Onset of disease(d)	Motor neuron loss(d)	Mean age at death(d)
Genomic hSOD1 G93A (G1)	18	90-120	>180	153-185
Genomic SOD1 G93A (G1H)	25	90	80	136
Genomic SOD1 G93A (G1L)	13	200	230	251
Genomic SOD1 G93A (G5/G5)	10	300	>300	>400

Despite a considerable amount of study, the alleged toxic gain of function in SOD1 remains scattered and elusive (85). The next segments aim to concise the argument of SOD1 PTM in pathological aging by prioritizing the following crucial aspects. What influences SOD1 to harbor toxic conformations particularly in the aging population, how these conformations structurally differ from each other, how they contribute to the disease pathogenesis in ALS and how the PTM SOD1 is implicated as a universal inducer of pathological aging specifically concerning ALS, AD, and PD affected patients.

1.9. SOD1 Post Translational Modifications by oxidative stress

An earlier study has claimed that SOD1 can adopt more than 44 conformations depending upon the variety and extent of its post-translational modifications, which harbor both physiological and pathological implications(86). Among them, oxidative stress-induced post-translational modifications are extensively studied and highly relevant to the study of accelerated aging in ALS patients.

1.9.1. Oxidation

Oxidation is a potential non-enzymatic post-translational modification, particularly about aging. It contributes to chemical modification at the susceptible side chains that shift the conformational stability towards the aggregation-prone state. Oxidized proteins are associated with numbers of age-related conditions such as ALS, Alzheimer's disease, Parkinson's disease, cataracts, rheumatoid arthritis, respiratory distress syndrome, cataracts, rheumatoid arthritis, and progeria (28) (87). Earlier findings indicated that the thiol group in cysteine residue is particularly susceptible to redox modification, and irreversible oxidation of a free single cysteine residue can substantially influence the native folding of a globular protein (88). Cysteine residues in proteins can exist as different redox forms among which thiol (-SH), disulfide forms are the most common, but derivatives with a higher oxidation state such as sulfenic (RSOH), sulfinic (RSO_2H), and sulfonic (RSO_3H) acid are being detected in a growing number of proteins. Sulfonic acid is the most highly oxidized species of thiols, and its formation is irreversible. Being an essential antioxidant SOD1 is least expected to be damaged by oxidative stress, yet oxidative stress-induced post-translational modifications of SOD1 are studied extensively mostly because of its structural conformation and close amalgamation with free radical homeostasis. The mass spectrometric analysis confirmed the oxidation of WT hSOD1 when exposed to H2O2. Electron capture dissociation (ECD) showed that cysteine 111 residue in SOD1 was initially targeted for oxidative modification to form irreversible sulfonic acid (80). It is understandable due to its native reduced thiol state (-SH) and surface localization on the protein, offering more exposure to environmental stress (29).

It corresponds to the findings in our lab, where pegylation reaction identified SOD1 oxidation in G93A transgenic mice. Besides, point mutation of the cysteine residues in SOD1 recognized cysteine 111 as the primary target of PTM by oxidation (29). Along with these, several other studies have addressed SOD1 oxidation at cysteine 111 residue as a preliminary stress response occurring to both wild type and mutant varieties(89),(90) (91)(92). Apart from cysteine 111, several other residues are described to

be modified post-translationally. For instance, high molecular weight SOD1 aggregates that increased up to 40-fold in aging cells of yeast mostly consisted of oxidized cysteine 146, histone 120, and histone 71 residues (93). Also, other forms of redox modifications such as carbonylation, glutathionylation, palmitoylation, and nitration are encountered at cysteine 111, cysteine 146, cysteine 57, and W32 residues of SOD1 (94)(95). Overall, oxidation of SOD1 is, so far, the most deliberate form of post-translational modification with potential consequences of misfolding and aggregate formation with pathological aging.

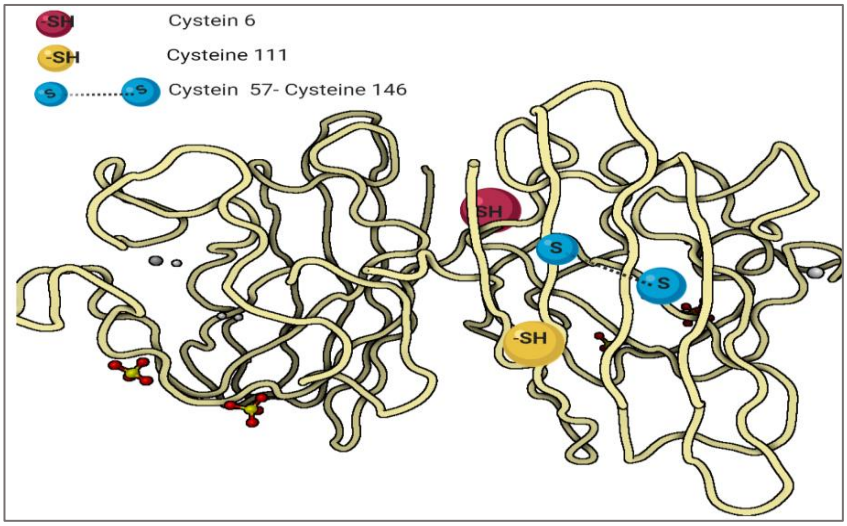

Figure 2. The image shows cysteine residues in SOD1.

Cysteine 57 and cysteine 146 (blue circle) form an intramolecular disulfide bond; cysteine 6 with intact thiol (-SH) group is buried under beta-strand. Cysteine 111 (yellow circle) is exposed on the surface close to the dimer interface.

1.9.2. Misfolding:

Misfolding refers to structural loosening of soluble SOD1 protein due to aberrant post-translational modification (28). Although the intramolecular disulfide bridge facilitates SOD1 stability, the oxidative modification could destabilize the protein and lead to misfolding before aggregate formation. Specific epitopes in SOD1 are identified to get exposed by misfolding, which are utilized to raise conformation-

specific antibodies(96). Human SOD1 contains a masked Derlin-1 binding region (DBR) expanding over amino acid residues 6-16 which upon exposure, captures the essential Derlin protein of EARD complex to induce ER stress(97). The unveiled DBR region in misfolded SOD1 which could be a potential therapeutic target for selective removal of aberrant SOD1 conformations without interrupting the native ones. Misfolded SOD1 may trigger detrimental consequences such as metal loss, disulfide bond reduction, monomerization, and further protein aggregation (95).

1.9.3. Aggregation and prion-like propagation:

In the context of the current study, aggregates refer to insoluble pathological inclusions in the brain tissues that are predominantly featured in the end stage of neurodegenerative diseases(98). Oxidatively modified SOD1 adopts a misfolded conformation that exposes the hydrophobic sequences of the protein to interact covalently with the exposed hydrophobic patches of other misfolded SOD1 in its vicinity. Such oligomers even offer covalent attachment to other native proteins and construct high molecular weight (HMW) aggregates (99).

Prion-like propagation of modified SOD1 is prominent at the end stage of diseases. The term 'Prion Like Propagation' is implicated conspicuously because prion diseases are infectious conditions, unlike neurodegenerative diseases such as ALS. However, post-translationally modified SOD1 shares some of the mechanical properties of infectious prion aggregates such as self-seeding and cross seeding with each other intracellularly and by the cell to cell transmissions(100).

It is hypothesized (73) that misfolded SOD1 acts as a structural template that interacts with its natural counterpart and induces the misfolding of captured protein in a template-directed reaction. Elongation of the misfolded SOD1 fibrils encounters spontaneous breakage that exposes new ends to allow prion-like replication of more native proteins and the spreading of the self-propagating core (99).

Also, the cell to cell transmission is another prion-like feature shared by modified SOD1 aggregates(97). The dissemination into the extracellular space might occur, followed by cell death or by exosome-mediated vesicular transport. The large aggregates stimulate micropinocytosis in neurons to expedite their propagation. Overall, once the protein is oxidatively modified to a critical limit, a chain reaction is instigated through subsequent misfolding, aggregation, and prion-like propagation, which may not precisely comply with the corresponding order.

1.10. Relevant evidence of SOD1 mediated accelerated aging in ALS

1.10.1. Modified SOD1 dysregulates ER and mitochondrial homeostasis

Misfolded SOD1 accumulates within the inter-membranous space of mitochondria and interacts with the voltage-dependent anion channel (VDAC1) and Bcl-2 on the mitochondrial outer membrane and induce mitochondrial dysfunction and apoptosis by demolishing VDAC1 conductance activity and converting Bcl-2 into toxic BH3 domain (72). Besides, there are multiple mechanisms through which misfolded SOD1 can induce ER stress. It interacts and blocks the function of coatomer coat protein II (COPII) that transports protein from ER to the Golgi apparatus (101), hence promote the sequestration of other aberrantly folded proteins. Again, SOD1 misfolding exposes Delrin binding sequence that interacts with Derlin-1 in ER. Derlin-1 is involved in the translocation of misfolded proteins from mammalian ER into the cytosol for degradation by the proteasomal or lysosomal system (102). By capturing the Derlin-1, misfolded SOD1 further induces ER stress. A recent study demonstrates that WT SOD1 aggregates in transgenic mice appear earlier than any other abnormal protein species during aging and have considered them as biomarkers for ER stress (73).

1.10.2. Induction of TDP-43 pathology

TAR DNA-binding protein 43 (TDP 43) is prevalently a nuclear protein that shuttles between nucleus and cytoplasm to cover a wide range of regulatory functions at the genomic level. For example, it

regulates the pattern of splicing in several transcripts that are substantially related to conditions like Cystic fibrosis transmembrane conductance regulator (CFTR), Fused in Sarcoma(FUS), alpha-synuclein (SNCA), Huntington (HTT), Amyloid precursor protein (APP) (103). Since 2006, pathological aggregates of TDP43 are detected from sALS, fALS, Frontotemporal dementia (FTD), dementia with Lewy bodies (DWLB), Alzheimer's disease, Parkinson's disease, and Huntingtin's disease (104)(105) affected brain.

In the ALS mouse model (G93A), a significant increase in TDP-34 pathology and hSOD1 aggregation coincided with the period of disease onset. Knockdown studies have confirmed that SOD1 mutation is responsible for TDP-43 pathology in familial ALS(104). Higashi et al. in 2010 implemented direct physical interaction of mutant hSOD1 with cytosolic TDP-43 and demonstrated that TDP43 intervenes at the SOD1 dimer interphase. Supposedly, the exposed hydrophobic surface of misfolded SOD1 can induce inappropriate interactions with other cellular components (106).

Oxidized and misfolded SOD1 were detected with TDP43 inclusions in the spinal cord of WT SOD1 transgenic mice and in vitro study with human neuroblastoma SH-SY5Y cells (106). Again, the addition of SOD1 aggregates in cell culture media with NSC-34 cells, induced TDP-43 aggregation inside the cells(107) (97). It also implicates the cell to cell transmission of SOD1 aggregates in disseminating the prion-like phenomena.

1.10.3. Invasion of the cellular stress response

SOD1 modification can interrupt cellular stress response by affecting the dynamics and functionality of stress granules (SGs) (108). These are membrane-less organelles in the nucleus and cytoplasm that resemble quick adaptive response to environmental stress and disassemble upon stress removal. They sequester and safeguard non-translating mRNA, translation repressors, RNA binding proteins (RBPs) with prion-like self-interacting domains(109). Stress granules also segregate mTORC1 component (110),

receptor for activated C kinase (RACK1)(109), TNF-α receptor-associated factor 2 (TRAF2) (111), Plasminogen activator inhibitor-1 (PAI-1) (112). Thus, stress-induced inflammatory, senescence, and apoptotic pathways are chiseled by them.

Interestingly, misfolded SOD1 is one of the components of such aberrant SGs. It was observed that SOD1 positive SGs were less mobile and lacking the typical liquid-like properties- fusion/fission (108). A depletion confirmed this in a mobile fraction of G3BP1 protein in SOD1 positive stress granule. The Ras GTPase-activating protein-binding protein (G3BP1) is a critical element in initiating and maintaining liquidity in the stress granule. It was previously reported that ALS linked mutant hSOD1 directly interacted and immobilized G3BP1(113). A later study showed that misfolded SOD1 interacts with various RBPs in the SGs to initiate a prion-like propagation process depending on the chronicity of oxidative stress (114).

1.10.4. Post-transcriptional dysregulation of mRNA

Specific loss of NF-L mRNA is a common characteristic in familial and sporadic ALS. NF-L is the foundation block of the heteropolymer built by the further addition of other subunits NF-M and NF-H. They function to provide structural support and mechanoresistance, form scaffolds for the organization of nucleus, mitochondria, and ER, play an essential role in vesicular transport, and maintaining axonal caliber (86). An initial study has demonstrated that mitochondrial SOD1(mtSOD1) destabilizes the NF-L mRNA by binding to its 3′- untranslated region (115)(86). Lack of NF-L hinders the subsequent polymerization and instigates perikaryal accumulation of remaining NF-M and NF-H subunits (116). Such neurofilaments aggregation collapses the vesicular transport in axon and is associated with neurodegeneration(117)(118). The remaining NFL are released into the circulation after neuronal loss, and hence, their serum levels inversely correlate with loss of brain volume associated with aging (117).

VEGF is a crucial angiogenic and neuroprotective growth factor that is declined with aging and is suggested as a therapy for old age (119). Similarly, misfolded conformations of mutant SOD1 bind and destabilize the VEGF mRNA, although a weaker interaction is seen with WT SOD1(120).

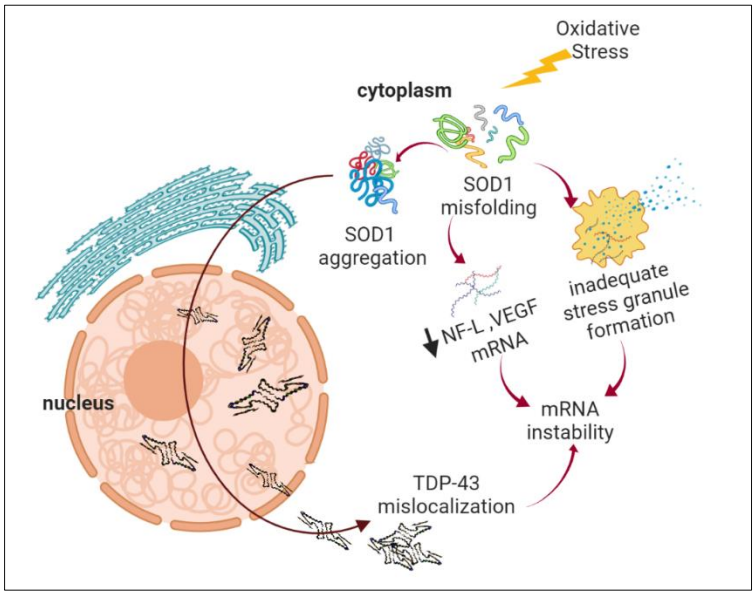

Figure 3. SOD1 Modification Renders RNA Instability

Oxidative stress-induced SOD1 modifications are responsible for direct mRNA instability by destabilizing the stress granules and reducing individual mRNAs. Also, it indirectly hampers RNA homeostasis by cytoplasmic mislocalization of TDP-43.

1.11. Modified SOD1 mediated protein aggregation in global neurodegeneration

Protein aggregation pathologies have gained importance concerning age-related neurodegenerative conditions since their topological distribution pattern and progression correlate with the clinical phenotypes of the patient (104). Until now, disease-specific protein aggregates are being formed by a particular pathological agent. Lately, aggregates composed of multiple pathological agents are being detected from patients phenotypically expressing one form of NDD (121). It raises the possibility of a

potential primary inducer, contributing to the neuropathological aging and illustrating diverse disease formats.

1.11.1. SOD1 aggregates in Parkinson's Disease:

Histopathologically, Parkinson's disease is characterized by the degeneration of dopaminergic neurons from substantia nigra and the presence of intranuclear inclusions known as Lewy bodies. It affects around 70% of dopamine secreting neurons in the substantia nigra by the end of life and deposition of Lewy bodies in many of the remaining neurons (122).

The Lewy bodies are hyperphosphorylated, misfolded, insoluble, fibrillated, and immuno-reactive alpha-synuclein inclusions along with ubiquitin (123). The central core (residues 61–95) of alpha-synuclein known as NAC (non-amyloid component) domain can form cross beta sheets and is comprised of highly hydrophobic sequences that contribute to its aggregation propensity and fibril formation (Berthold et al. 2016). Moreover, alpha-synuclein in the cytoplasm is an intrinsically disordered protein that lacks a single stable 3-dimensional (3D) structure. Thus, it is susceptible to PTM by phosphorylation of its serine 129 residues in the C terminal domain and also contributes to abnormal cluster formation(124). Alpha-synuclein induced protein aggregates to propagate their toxic effects by synaptic vesicle impairment, mitochondrial dysfunction, ER stress, oxidative stress, autophagy, and lysosomal pathway dysfunction (125). Notably, apart from their widespread intra-neuronal distribution, they are also deposited in oligodendrocytes and astrocytes in the PD affected brain (126). Compared to their widespread distribution, the pattern of neuronal involvement is quite selective in PD, indicating the possible involvement of other influences in the disease pathology. On top, therapies targeted to the pathways of alpha-synuclein toxicity brought disappointing results in clinical trials(127), supporting the involvement of additional neurotoxic mechanisms in PD.

The vulnerable regions of Parkinson's disease, such as SN, LC, are characterized by oxidative stress and the regional copper deficiency(128)(129)(130)(131). In this context, enhanced carbonylation of SOD1 is found to be associated with Parkinson's affected brain(132). Further progress was made by identifying direct physical interaction between the alpha-synuclein and SOD1 in the mouse brain and human erythrocytes (133). This interaction was found to ameliorate their respective oligomerization process. However, to what extent the SOD1 proteinopathy is distinct from typical synucleinopathy and whether the SOD1 interaction plays a role in exacerbating the disease process or act as a potential mechanism in the initiation of the disease pathology, is still unknown.

The above concerns were elaboratively studied by Trist et al. in 2017, where, they demonstrated that the SOD1 aggregates in the PD brain are closely associated with the regional pattern of neuronal loss, for example, SN has shown an eight-fold higher density of SOD1 aggregates compared to the age-matched controls and a significantly higher density than the non-degenerating region of the same PD affected brain. The structural composition of these aggregates revealed that only 50% of the SOD1 aggregates in this PD brain were negative for alpha-synuclein, but positive for both CCS and ubiquitin(127). It was not surprising as because previous data ensured that oxidative modification of SOD1 suppressed the components of the proteasomal system in an age-dependent manner(134). The co-aggregation of ubiquitin with SOD1 could reflect the saturation of damage clearing mechanism

The question remains in the first place is how the SOD1 gets modified in Parkinson's affected brain. Interestingly, copper and iron redox dysregulation are emphasized as significant contributors to disease pathology (135). Long term occupational exposure to copper increases the risk of PD (130). Free transition metal like copper is known for its toxicity, especially by producing deleterious hydroxyl free radicals by Fenton reaction and converting low reactive molecules like flavin, ascorbate, lipid peroxides, thiols to highly reactive species (136). Oxidative stress-induced by free copper can directly abet the SOD1 and alpha-synuclein aggregation. Secondly, copper-deficient SOD1 itself shows a 66% reduction

in the enzymatic activity in metal deficient SN that further propagates the stress level in PD affected brain(127). Copper dysregulation can potentially be more toxic to SOD1 than alpha-synuclein in the context of Parkinson's disease. Furthermore, both copper deficit and misfolded SOD1 appears in SN in the early stage of the disease pathology, even before the onset of neuronal loss and similar SOD1 aggregates are also detected in the SN and LC of ALS affected brain (133)(127), suggesting the vulnerability of these regions to pathological alterations of SOD1 protein.

1.11.2. SOD1 and Alzheimer's disease

Alzheimer's disease, the most common cause of dementia in the elderly (137), is characterized by extracellular beta-amyloid plaque (Aβ) and intracellular neurofibrillary tangles, consisting of the microtubule-associated protein tau (138). Initially, there is increased production and decreased breakdown of beta-amyloid, which subsequently accumulates and forms oligomers (139). Aβ fragments are generated through sequential degradation of amyloid precursor protein (APP), which is removed by microglial phagocytosis, receptor-mediated internalization by astrocytes and also protease neprilysin and which is imperative in the degradation of Aβ fragments (140). According to the amyloid hypothesis, the formation of Aβ is the pathological phenomenon that is associated with the onset and progression of the disease (141). In contrast to Aβ, tau deposits better correlate with the disease course or the degree of cognitive impairment (142). The primary prevention trials thus target the Aβ pathology (141). However, at the incipient stage of the disease, the Aβ accumulates intracellularly and hence, is described as "a sign of worse things to come" (143). What triggers the initial Aβ accumulation to progress into the advanced stages of extracellular plaque formation is still unknown (144). To arrest the disease before commencement, we must focus on the preliminary events that precede the Aβ aggregation.

Earlier studies showed that SOD1 aggregation in the ALS mouse model was aggravated by amyloid-beta (Aβ) overexpression. For instance, Aβ-SOD1 aggregates are found in H4 cells (human neuroglioma

cells) with a higher affinity of Aβ to mutant SOD1 than to wild-type SOD1 (145). Another study, as mentioned earlier, showed the substantial existence of SOD1 aggregates in the brains of AD and PD patients, as well as in healthy controls (146), thus, expanded the role of SOD1 to non-ALS disease.

Considering the association of oxidative stress in AD pathology (147) and SOD1 being a crucial player of endogenous defense against free radicals (148), our lab argues that the non-native conformations of SOD1 could be the primary seeding hub for the Aβ peptide fragments. Considerable evidence implies that AD damage arises primarily from small oligomeric amyloid forms of Aβ peptide, but the precise mechanism of pathogenicity remains to be established(149). It is in the general well established that any type of amyloid fibril formation is initiated by exposure of small aggregation-prone segments (150). As mentioned earlier, cysteine 111 mediated oxidation can lead to misfolding of SOD1(29). Misfolded protein exposes DSVISLS and GVIGIAQ segments in the C terminus, which are typically buried in the dimeric interface of functional SOD1(151). These segments can form tightly packed beta-sheets with steric zipper interfaces, which are characteristics of the amyloid fibrils. It was reported that SOD1 has a specific Aβ binding region with which it could interact with Aβ protein and initiate further aggregation and cell death (149). This Aβ binding region is cloistered by hydrophobic interaction in native SOD1, supporting the existence of aggregation-prone segments in the C terminus, which lies in the dimeric interface of functionally active SOD1. However, the gene for SOD1 and amyloid precursor protein both are expressed by chromosome 21, and the upregulation of SOD1 is proven to be protective against oxidative stress from Aβ toxicity(84).

In contrast, SOD1 overexpression is associated with oxidative modification of itself and, in turn, induces the aggregation pathology (152). Unpublished data from Dr. Kong's lab shows that oxidized and misfolded SOD1 promotes intracellular Aβ aggregation, both in vivo, in vitro, and cell-free culture medium. Double transgenic mice ($APP^+/G37R^+$) have much aggressive expression of Aβ aggregates compared to APP^+ transgenic mice. Besides, phenotypically, up to 50%-60% of all ALS patients exhibit

cognitive defects collectively known as the frontotemporal spectrum disorders of ALS (ALS-FTSD) (153), suggesting SOD1 with non-native confirmation can induce Aβ aggregation. Besides, this phenomenon also suggests that SOD1 overexpression in response to oxidative stress maintains a critical level beyond which SOD1 aggregate formation overhauls its enzymatic activity and propagate in a prion-like manner.

1.12. Summary

In this chapter, I have portrayed various aspects of aging that are crucial to the molecular research of aging-associated conditions. Also, it elaborated on the possible link between the post-translational modification of SOD1 and ALS in the backdrop of aging, considering the brief life span associated with ALS. Chapter 2 aims to determine the role of oxidatively modified SOD1 as a mechanism of aging in ALS.

2. Chapter: Hypothesis, Objective, Method & materials, Discussion

2.1. Hypothesis

Oxidatively modified SOD1 is a mechanism of pathological aging in ALS.

2.2. Objectives

- To establish the correlation between oxidized SOD1 and pathological aging
- To observe the knock down effect of oxidized SOD1 on aging.

2.3. Materials and Methods:

2.3.1. Mice and Tissue Preparation

Transgenic mice carrying human G93A mutant SOD1 (B6. Cg-Tg (SOD1*G93A) 1Gur/J) and human G37R mutant SOD1 (B6. Cg-Transgenic (SOD1*G37R) 42Dpr/J) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The offspring came by crossing these mice with C57BL/6 background for at least four generations. Genotyping of DNA from ear biopsies by PCR was done by adopting a Jackson Laboratory protocol. The Central Animal Care Services (CACS), the University of Manitoba, maintained the animal colonies in an environment free of pathogens. All procedures used in this study followed the guidelines established by the Canadian Council on Animal Care according to standard animal care protocols.

In contrast to the C57BL/6J background, where 50% survival is seen at 157.1+/-9.3 days, the G93A mice have an abbreviated life span: 50% survive at 128.9+/-9.1 days. Before the onset of the disease, the animals are healthy. Considering their average life span of 160 -180 days, female and male G93A mice were sacrificed at their young age (~50 days), middle-age (~day 100 and 130), and old ages(~day 150) where each of the four age groups had 3 mice. The age predictions were based on our previous experience of handling the animal models of ALS as well as the previous publications. The animals were perfused

with 20 ml phosphate-buffered saline (PBS) through the transcardial route following anesthesia. Spinal cord tissues were collected for further analysis.

2.3.2. CT-4 peptide Treatments:

To determine the effect of SOD1 modification on pathological aging, we knocked down the misfolded SOD1 in the G37R transgenic mouse model by injecting CT4 peptide for 30 consecutive days. Beginning at 70 and 120 days of age, 3 animals in each age group, were treated with an intraperitoneal injection of CT4 peptide at a dose of 20 µg/kg. The animals were anesthetized, and the tissues were collected at 100- and 150-days age of treated mice (3 in each age group) to be compared with an equal number of age-matched non-treated animals.

2.3.3. Transient Transfection of hSOD1-WT/SOD1-G93A in N2A cells:

2.3.3.1. Plasmid Construct and Peptide:

Wild-type human SOD1 (hSOD1-WT) and SOD1-G93A constructs were constructed in the same manner as previously reported (192). The RT-PCR cloned hSOD1-WT gene was from total RNA extracted from the hSOD1-WT transgenic mouse. The wild-type template for PCR amplification uses the following primers: 5'- GCGCGCGTCGACAAGCATGGC-3' (forward), 5'-GCGCGCGTCGACGCTTGGGGCGATCCCAAT-3' (reverse). Similarly, the SOD1-G93A gene was cloned by RT-PCR from total RNA extracted from SOD1-G93A transgenic mouse. The G93A template for PCR amplification uses the following primers: 5'CTGCTGACAAAGATGCTGTGGCCGATGTGTC3'(forward),5'GACACATCGGCCACAGCATCTTTGTCAGCAG-3' (reverse). All the plasmid constructions were verified by automated sequencing. DCT4-CTM and mDCT4-CTM plasmids were constructed by inserting the DCT4-CTM or mDCT4-CTM coding sequence into the pEGFP-N2 vector (Addgene) using BglII and EcoRI restriction sites

(restriction enzyme and buffer from Thermo). The DCT4-CTM and mDCT4-CTM coding sequence was prepared by annealing custom-designed oligonucleotides (Integrated DNA Technologies). CTM control plasmids were obtained from Dr. Yutian Wang (University of British Colombia, Vancouver, Canada)

2.3.3.2. Cell Culture and Transfection:

The adherent N2a cell line was maintained routinely in DMEM (Sigma) supplemented with 10% fetal bovine serum and 1% Penicillin (- Invitrogen) at 37 ℃ in a humidified incubator with a 5% CO_2 atmosphere. Transfections for transient expression of hSOD1-WT/SOD1-G93A constructs (1 µg total DNA/2 x 10^5 cells) were performed using FuGENE 6 transfection reagent (Promega, Madison, WI) following the manufacturer's instructions. Briefly, N2a (mouse neuroblastoma cell line) were recovered from liquid nitrogen and cultured at least one week before the transfection to make sure that cells were growing accordingly. A day before the transfection, N2a cells were seeded in a T25 flask at 2× 10^5 cells/ml in 5 ml of DMEM medium complete culture medium. Twenty-four hours later, cells were transfected with plasmids. FuGENE transfection reagent was added to the Opti-MEM medium (Invitrogen) and incubated for 5 minutes. After incubation, an appropriate amount of DNA was mixed with FuGENE/Opti-MEM to achieve the ration of the reagent to DNA (3:1) and further incubated for 20 min at room temperature. After incubation, the mixture was added to the cells, and the cells were maintained in the incubator for 48 hours, followed by sub-culturing with fresh media containing G418 (1000µg/µl) to kill the non-transfected cells selectively. Then the cells were routinely sub-cultured for three weeks. The group transfected with G93A plasmid were further divided into two groups to be transfected for the second time with DCT4-CTM and mDCT4-CTM plasmid after one week of their initial transfection and was maintained by sub-culturing every 3rd day for the next two weeks. The cells from each group (WTsod1, G93A, G93A+CT4, G93A+Mct4) were harvested for IF and western blot analysis at one week, two weeks, and three weeks interval.

2.3.4. Bacterial Transformation

2.3.4.1. Growth and Induction of Cells

The BL21 strain of E. coli bacteria were used for transformation. We had inserted the CT4 (FLYRWLPSRRGG) sequence into the PGEX-4T-1 backbone to generate pGEX-4T-1-CT4 (unplugged extracellular domain expressing vector). The vector was Ampicillin resistant. They were mixed 0.5 µl of pGEX-4T-1-CT4 and PGEX-4T-1 (control) into 50 µl of competent cells separately by flickering with hands. For thirty minutes, the mixture was incubated on ice with subsequent heat shock at 42° C. for 45 seconds. 500 µl SOC media was added to the bacteria and were grown at 37° C in a shaking incubator for 45 minutes at 200 RPM. On agar plates, 50 µl from each tube was added by spreading with T tubes, incubated overnight at 37° C in a shaking incubator at 200 RPM.

Several colonies containing PGEX-4T-1-CT4 were inoculated in 2 ml of LB (Luria Bertani) medium added with Ampicillin (100 µg/ml). For comparison, control tubes were inoculated with bacteria transformed with parental pGEX plasmid. The inoculum was grown overnight at 37°C with vigorous shaking. The next morning, cultures were diluted into 2x LB medium (1:100) (i.e., 1980 µl LB+20 µL bacterial culture) and further shaken vigorously at 37°C for 3 hours. The fusion protein expression was induced by adding 2 µl of IPTG diluted in LB medium to achieve 100 µM concentration. The concentration was optimized by experimenting with different concentrations of IPTG (Isopropyl β-D-1-thiogalactopyranoside), followed by western blot analysis. Next, the tubes were shaken in an incubator for an additional 2 hours. The liquid cultures were transferred to the microcentrifuge tubes and centrifuged for 5 seconds in 2000 RPM at room temperature (RT) to get the pellet cells by discarding the supernatants. After washing the pellets with wash buffer thrice, each of them was resuspended in 50 µl of ice-cold 1x PBS for each ml of culture that was centrifuged (here, total 2 ml)

2.3.4.2. Cell Lysis

5 mg/ml lysozyme solution was prepared with water to add 2 µl (0.1 mg/ml) in each 100 µl of cell suspension. Vortexed the tubes gently to disperse the lysosome and incubated at room temperature for 5 minutes. The samples were sonicated three times with 3 seconds sonication and 30 seconds gap at each time. Next, centrifuged for 10 minutes at 2000 RPM and RT. The supernatants were collected separately for the subsequent part of the experiments. The supernatants are divided into two groups, one with GST-CT4 and another with GST only (control).

2.3.4.3. GST Pull-Down Followed by Western Blot Analysis

100 µl of each of the supernatants were incubated with 50 µl of 25% glutathione Sepharose (25% slurry was made using TBS buffer) (GE 45000139) overnight in the +4°C rotator. It bound the beads with GST and GST-CT4. Next, we washed the mixture and centrifuged at 2000 RPM for 5 minutes to get rid of the unbound glutathione beads, and the pellets were incubated with G93A transfected (48-hour post-transfection, 1-week post-transfection) N2a cell lysates (100 µl for each) for an hour in +4°C rotator.

The incubated samples were washed thrice, and both the pellets and supernatants were collected for subsequent western blot analysis by adding 2x loading buffer and subsequent denaturation at 95°C. Two separate blots were incubated with two different SOD1 antibodies (MA1-105, AB52950) to confirm the result.

2.3.5. Western Blotting

Collected mice tissues (spinal cord) homogenized in 10 volumes of IP lysis buffer (Pierce, Cat# 87787, Thermo-Fisher) mixed with a 1% (v/v) cocktail. The homogenates were centrifuged at 21,000 × g for 30 minutes at 4 ℃ to isolate the supernatants.

The cellular samples in the flasks were washed thrice in ice-cold PBS, followed by scrapping with an IP lysis buffer and was transferred into a microcentrifuge tube. The cell suspension was maintained by constant agitation for 30 minutes at 4 ℃ with subsequent centrifugation at 21,000 × g for 30 minutes, and supernatants were collected for further analysis. BCA Protein Assay Reagent (Pierce, Rockford, IL, USA) was utilized to assess protein concentration. Samples were denaturated by boiling in Laemmli sample buffer containing 2.5% β-mercaptoethanol for 5 minutes, and 10 μg of sample proteins were separated by 12% TGX Stain-Free polyacrylamide gels (Cat #1610185, BioRad), and transferred to PVDF membrane by Trans-Blot Turbo Transfer System (Bio-Rad). Membranes blocking was done with 5% (w/v) fat-free dry milk in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween-20 for 1 hour and incubated with primary antibodies overnight at 4 ℃ (See table 3 for the detail of primary antibodies). Appropriate secondary antibodies were used to incubate the blots for 1 hour at room temperature. The protein bands were visualized in the imaging machine (ChemiDoc MP, imaging system, Bio-Rad) with the enhanced chemiluminescence reagent (ECL Prime, GE Healthcare, Cat# RPN2232) (ChemiDoc MP, imaging system, Bio-Rad).

2.3.6. SA-β-Gal staining

Beta-Gal staining was performed using Senescence –galactosidase staining kit (Cell Signaling Technology) following the manufacturer's protocol. In brief, cells grafted on the coverslips in the 24 well plates were fixed with the fixative solution provided by the company followed by overnight incubation with β-Gal staining solution (pH-6) at 37°C in a dry incubator (no CO_2). While the β-

galactosidase was still on the plate, the cells were examined under a microscope (200X total magnification) for the development of blue color.

2.3.7. Immunocytochemistry (ICC)

Poly-D-Lysine (PDL) coated coverslips were used to grow the cells, which were fixed in 4% paraformaldehyde in PBS pH 7.4 for 15 minutes at room temperature and then were washed thrice with PBS. Samples were incubated for 10 minutes in PBST (PBS containing 0.25% Triton X-100) for improving the penetration of the antibody. Then the coverslips were washed thrice with subsequent incubation in 1% BSA in PBST for 30 minutes to block unspecific binding of the antibodies. Samples were incubated with primary antibodies in 1% BSA overnight at 4℃, as mentioned in Table 3. Secondary antibodies were applied after three washes and incubated in the dark for 1 hour at room temperature. After three final washes, the cell nuclei were counterstained with Hoechst 33342 (Calbiochem), and then coverslips were mounted with a drop of fluorescence mounting medium (Dako North America, Inc. Carpinteria, CA 93013, USA). Carl Zeiss AxioImager Z2 microscope and processed with Zen Pro imaging software (Zeiss, Germany) was used to capture fluorescent images.

Primary Antibodies:

Table 3: lists of primary antibodies

Antibody	Host Species	Application (Dilution)	Source	Cat #
SOD1 (8B10)	Mouse	WB (1:1000)	ThermoFisher	MA1-105
Human SOD1	Rabbit	WB (1:1000)	Abcam	AB52950
Misfolded SOD1 (C4F6)	Mouse	IF (1:200)	MediMabs	MM-0070-2P
P^{53}	Mouse	WB (1:500)	Santa Cruz	SC-126
P^{16}	Mouse	WB (1:500)	Santa Cruz	SC-166766
β-Galactosidase	Mouse	WB (1:500)	Santa Cruz	SC-65670

2.3.8. Statistical Analysis:

Data are presented as the mean ± SD. For statistical comparison between the two groups, Student's t-test was used. Multiple comparisons were performed by one-way ANOVA, followed by Bonferroni's multiple comparisons test. Differences were considered to be statistically significant (*) when $P < 0.05$.

2.4. Results

2.4.1. G93A transfected N2a cells exhibited senescence and reduced proliferation

N2a cells were transiently transfected with G93A and wild type human SOD1 (hSOD1-WT) plasmid constructs in separate flasks (T25 flasks). 48- hours post-transfection, the transfected cells were cultivated by regular sub-culturing with fresh media every 3rd day, consequently for 3 weeks. Each time, the cells were sub-cultured, G-418 (1000 µg/ml) was added to the DMEM media for negative selection of removing the non-transfected cells. The effective concentration of G418 was obtained from the WST analysis of N2a cells, as mentioned earlier (Figure 2.1). While sub-culturing, the cells were collected at 48-hour, at 1-week, 2-week, and 3-week intervals post-transfection for subsequent experiments.

Transfected N2a cells were fixed for immunofluorescence study with human SOD1 antibody (AB52950) revealed that almost all the cells were resistant to G-418 even at the end of 3rd wk following the transfection (figure 2.3), allowing us to exclusively study the consequence of G93A and hSOD1-WT plasmid expression without clonal selection. The addition of external stimulants such as oxidative stress-inducing agents was avoided so that we could determine the effect of hSOD1 plasmids on cellular aging solely.

The immunofluorescence (IF) study showed that the Ki 67 positive cells (red fluorescence) were strikingly alleviated in the G93A group (l, o, r) compared to the WTSOD1 group (c, f, i) over the 3 weeks of the experiment. Ki 67, as a proliferation marker, is the most widely used for subjective assessment of

cellular proliferation in both aging and cancer studies. A significant relevance of Ki 67 to our study is that it reveals the cellular proliferation in a graded manner throughout the cell cycle. For instance, a potentially low Ki 67 is due to the continuous degradation of Ki 67 when the cells are residing at the G_1 /G_o stage. Contrary, Ki 67 increases throughout the S and G2 phase and reach a peak in actively proliferating M phase of the cell cycle (161). The dynamicity of the Ki 67 throughout the cell cycle sets a demarcation between proliferation and quiescence.

Again, these two groups were comparatively analyzed at three weeks, based on the percentage of cells positively stained with β-galactosidase. The finding complemented the KI-67 expression pattern by exhibiting a notable increase of β-gal positive cells in the G93A transfected group (2.6). To further confirm cellular aging in the G93A group, WB analysis was done, which revealed a radical increase of senescence in G93A groups (figure 2.7 b, d, f, h). In contrast, the WTSOD1 transfected cells were indifferent throughout the experimental period (figure 2.7 a, c, e, g).

Collectively, we observed that the G93A transfected cells were experiencing accelerated aging, whereas the aging process was more subtle and physiologically relevant in WTSOD1 transfected cells.

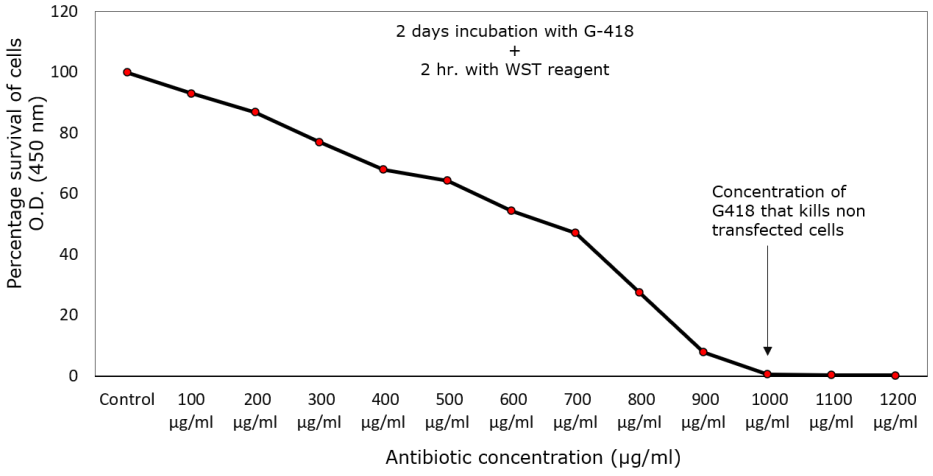

Figure 4. Effective concentration of G418 was optimized by WST analysis.

An equal number of cells (10,000/well) were seeded in 96 well plates. The next day media were replaced by G-418 containing DMEM media and cultivated for 2 days with subsequent WST analysis on the 3rd day using a 96-well plate reader. The experiments for each concentration were done in triplicates and repeated 5 times. The controls without any G-418 were incubated for the same period with WST reagents. The line graph showed the percentage of cell survival in Y-axis against the different concentrations of G-418 in X-axis to mark the minimum concentration of G-418, which could effectively kill all the non-transfected cells.

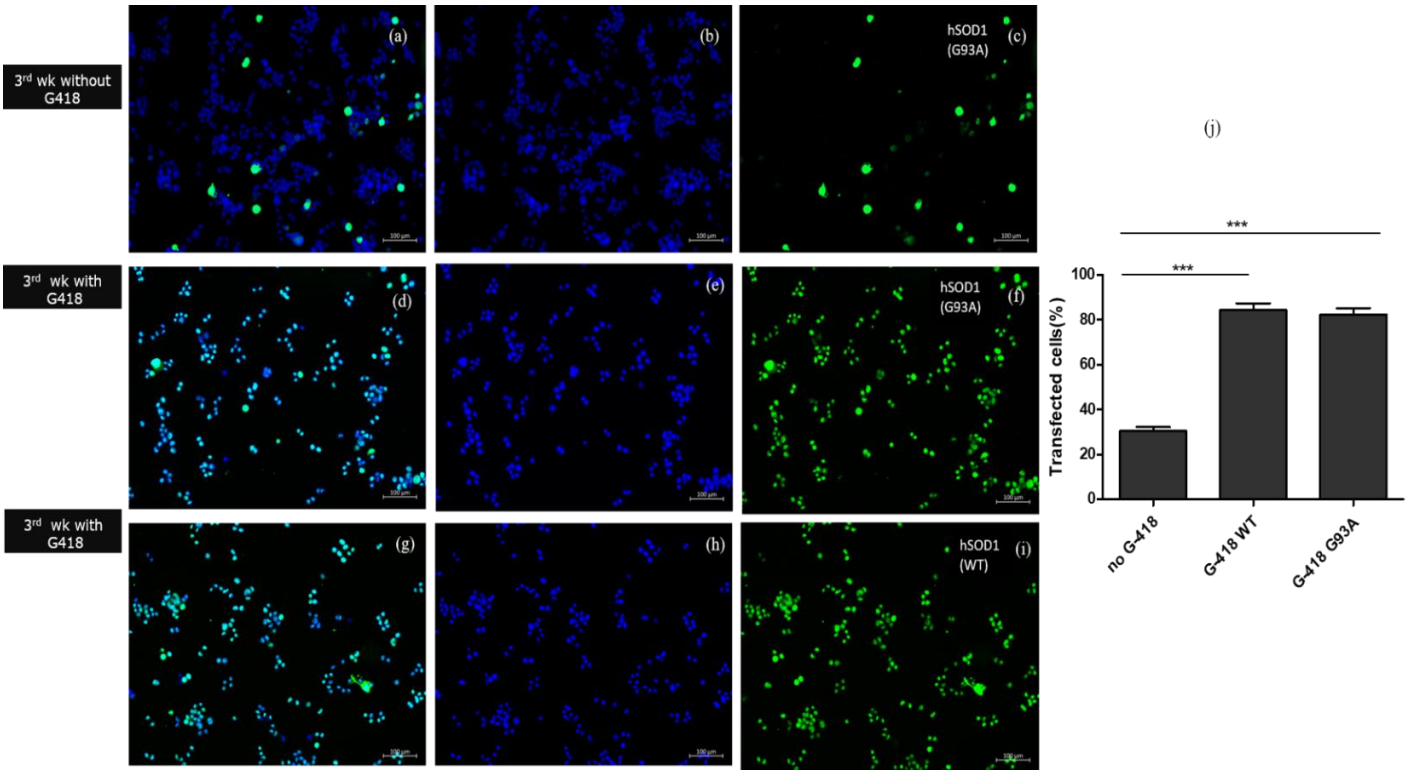

Figure 5. The expression of hSOD1 plasmids are substantially enhanced in transiently transfected N2a cells by negative selection with G-418.

The addition of G-418 (1000 µg/ml) effectively diminished the growth of non-transfected cells. Similar expression of hSOD1 was evident in G93A (d-f) and WTSOD1 (g-i) groups, which were treated with

G418 over the extent of 3 weeks. G93A transfected Control group (a-c) was cultivated concomitantly for the same duration without G418 that exhibited significantly lower expression of hSOD1. One-way ANOVA, followed by Bonferroni's Multiple Comparison Post-Test, was performed to compare the groups. The asterisk (***) indicates a significant ($P < 0.0001$) difference between the groups with and without G-418. Means ± SEM, n=15, n represent images captured from 3 independent cultures. Scale bar =100 µm.

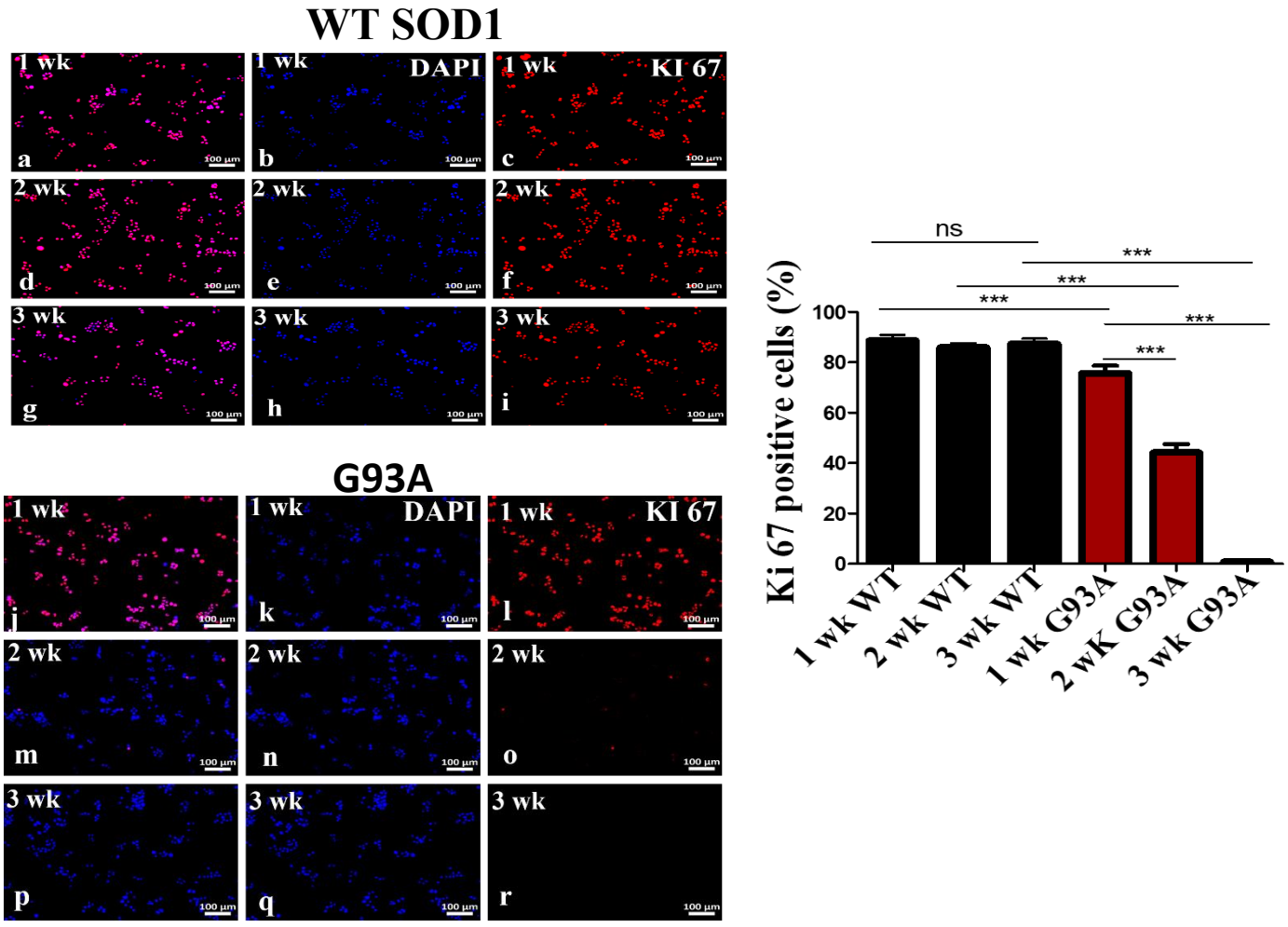

Figure 6. A potential diminution in cellular proliferation was marked by Ki 67 expression in G93A transfected N2a cells.

Representative images are of the immunofluorescence staining of N2a cells transiently transfected with plasmids encoding G93A and WT SOD1. The red fluorescence signal indicated Ki 67 protein (red) with a nucleus dyed by Hoechst (DAPI). The (a-c), (d-f), and (g-h) panels respectively marked the cells of WTSOD1 groups after 1,2 and 3 weeks of cultivation with G-418. Again, (j-l), (m-o), and (p-r) panels accordingly identified G93A cells after 1,2 and 3 weeks of transfection with G-418. The Ki 67 expression in the WT SOD1 group in c, f, and i panels were comparable, whereas l, o, and r panels displayed a considerable decline in cellular proliferation in the G93A group. One-way ANOVA, followed by Bonferroni's Multiple Comparison Post-Test, was performed to compare the groups. The asterisk (***) indicates a significant ($P < 0.0001$) difference between the groups. Means ± SEM, n=15, n represent images captured from 3 independent cultures. Scale bar=100 µm.

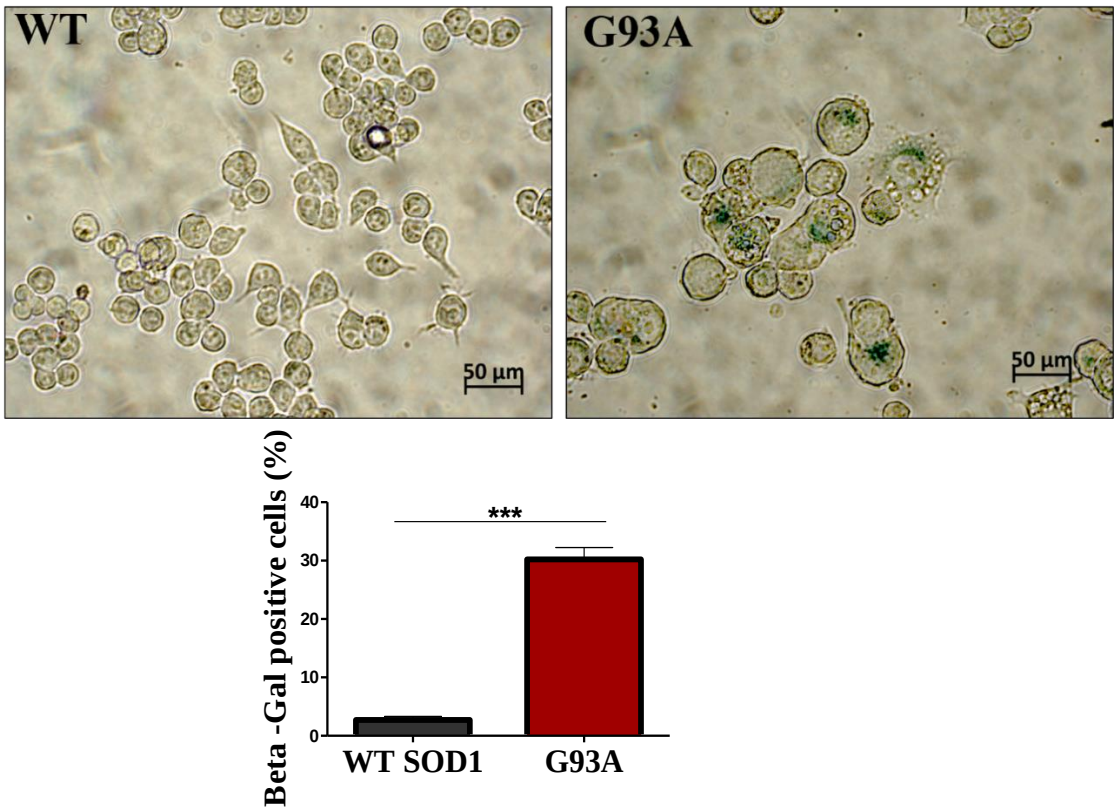

Figure 7. β-gal staining in the G93A group showed a substantive increase compared to the WT group 3 weeks post-transfection

Implicating phase-contrast microscope, SA-β-gal is demarcated by characteristic intracellular blue staining, pointed in the images by blue arrows. For statistical analyses, Unpaired t-test was performed to compare the groups. The asterisk (***) indicates a significant ($P < 0.0001$) difference between the groups with and without G-418. Means ± SEM, n=15, n represent images captured from 3 independent cultures. Scale bar=50 µm.

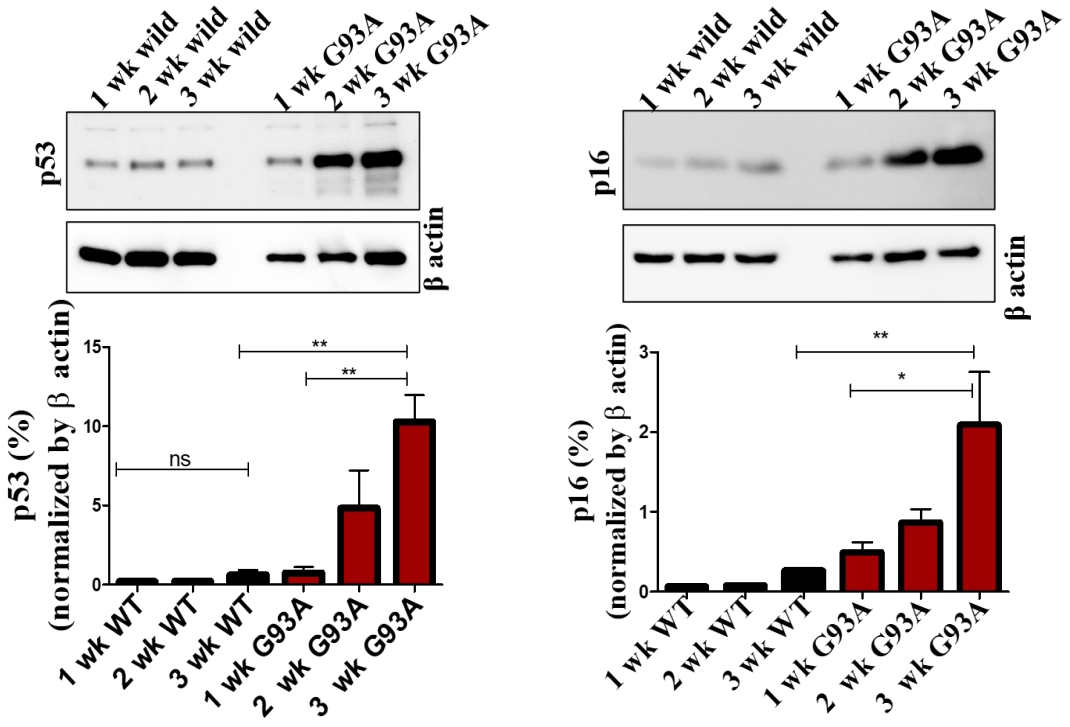

Figure 8. cellular senescence significantly increased in G93A cells over the 3 weeks of transfection. One-way ANOVA followed by Bonferroni's Multiple Comparison Post-Test were performed to establish significant differences among the groups. The asterisk (*) indicates a significant ($P < 0.05$) difference between the youngest (50 days) and the oldest group (150 days) of mice. Each experiment was repeated independently 3 times. Bars represent mean ± SEM, n=3 mice per group.

2.4.2. Oxidative modification of hSOD1 surged in G93A mice models in an age-dependent manner

An equal volume of spinal cord tissue lysates collected from four different age groups of G93A transgenic mice was incubated with 3 mM of MalPEG (mono-methyl polyethylene glycol 5,000 2-maleimidoethyl ether, Sigma) for 1 hour in the room temperature. Subsequently, the reaction was abrogated by adding 5% β-mercaptoethanol, followed by denaturation at 95° C and running samples in 12% SDS-PAGE gel (10µg/lane). The blot was incubated overnight with an anti-SOD1 antibody (8B10), which could bind with both human and mouse SOD1. Due to their structural dissimilarities (i.e., cysteine 111 residue replaced by serine) and different molecular weights, distinct bands at 17 kDa, and 15 kDa locations are indicative of human and mouse SOD1, respectively. To observe the aging-associated alterations, we have included four different age groups of G93A mice- 50 days, 100 days, 120 days, and 150 days with 3 animals in each age group.

On the other hand, MalPEG, as previously described (29), is an alkylating agent linked with 5 kDa PEG that is widely utilized to detect oxidative modification of sulfhydryl groups in specific proteins in western blot format. MalPEG bonds covalently to the sulfhydryl group (-SH) of the cysteine residues and the MalPEG-protein conjugates are detected in the western blot as additional bands, 5-10 kDa higher (per modification) than the native location.

Previous publications from our lab established that MalPEG modification of hSOD1 was at first place Cysteine 111 specific due to its greater accessibility on the surface of the protein. In the current experiment, hSOD1(MP)1 band (figure 2.6) indicated the hSOD1 in its natively reduced forms (-SH) that were modified each by one molecule of MalPEG. Such modifications, detected by apparent band shifts in the western blot, were reduced in older mice. Besides, the hSOD1(MP)2 represented aberrantly formed hSOD1 dimers that are accessible to two molecules of MalPEG. The result showed that the cysteine 111 oxidation in SOD1 progressively increased with age and displayed a notable rise in the oldest group compared to the youngest one.

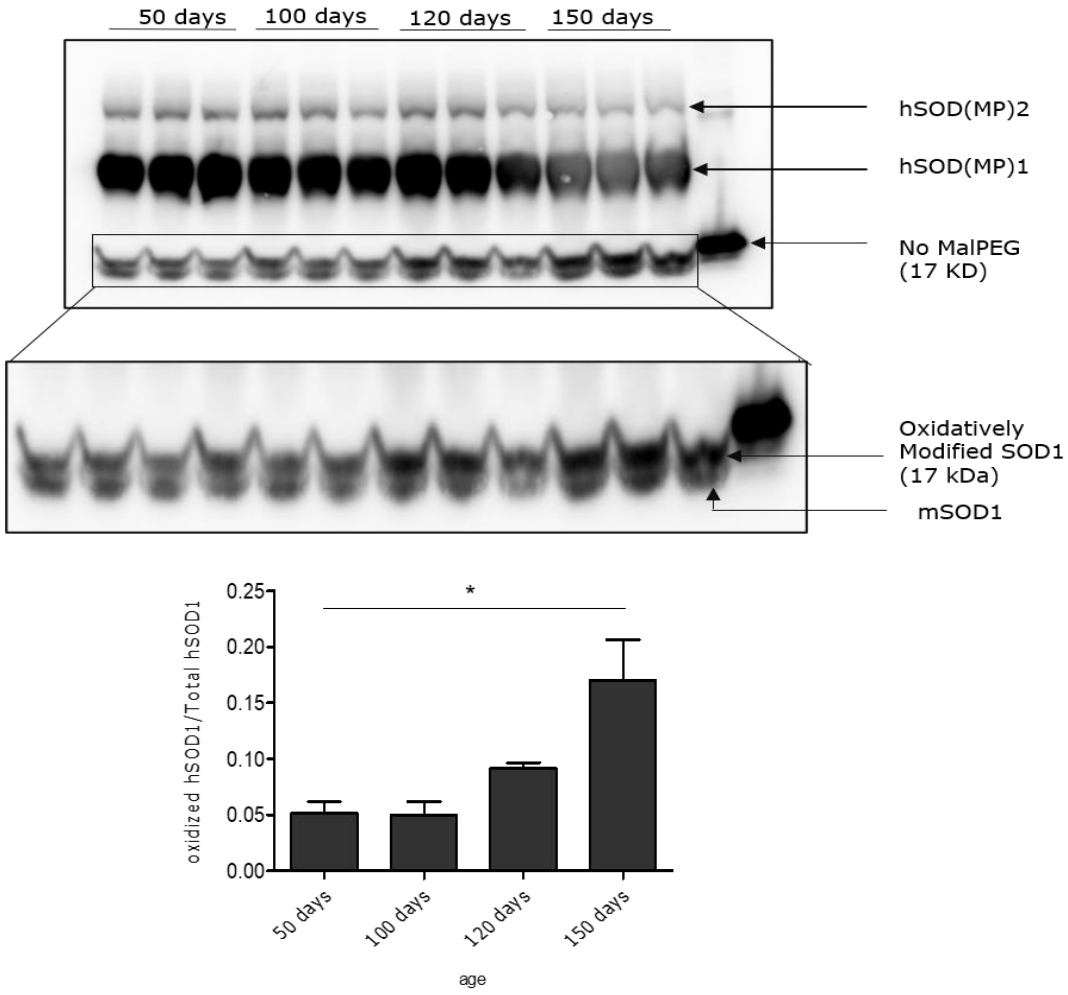

Figure 9. MalPEG inaccessible oxidized SOD1 increased in an age-dependent manner

(a)In SOD1 (MP)1 band group, the Cysteine 111 (-SH) in its native form is modified by one molecule of MalPEG. Cysteine-MalPEG conjugation was reduced with a parallel and significant rise of oxidized SOD1 (at 17 kDa location) in the oldest group compared to the 50 days old group of mice. The MalPEG inaccessible hSOD1 was quantified as oxidized hSOD1 immunoreactivity per total hSOD1 immunoreactivity. One-way ANOVA, followed by Bonferroni's Multiple Comparison Post-Test, was performed to establish significant differences among the groups. The asterisk (*) indicates a significant ($P < 0.05$) difference between the youngest (50 days) and the oldest group (150 days) of mice. Each experiment was repeated independently 3 times. Bars represent mean ± SEM, n=3 per group.

2.4.3. Aging-associated senescence markers in G93A model synchronized with the gradual age-dependent increase of oxidized hSOD1

Next, we have utilized the lysates from the same spinal cord tissues for quantitative assessment of the senescence markers p16INK4a, p53, and SA-β-gal by western blot analysis. Similar to the oxidized SOD1 in the previous result, the markers gradually escalated with age. The result implicated a concomitant rise of modified hSOD1 and senescence markers in an age-relevant manner, which could be interpreted as two independent processes or inter-related mechanisms influenced by aging-associated modifications.

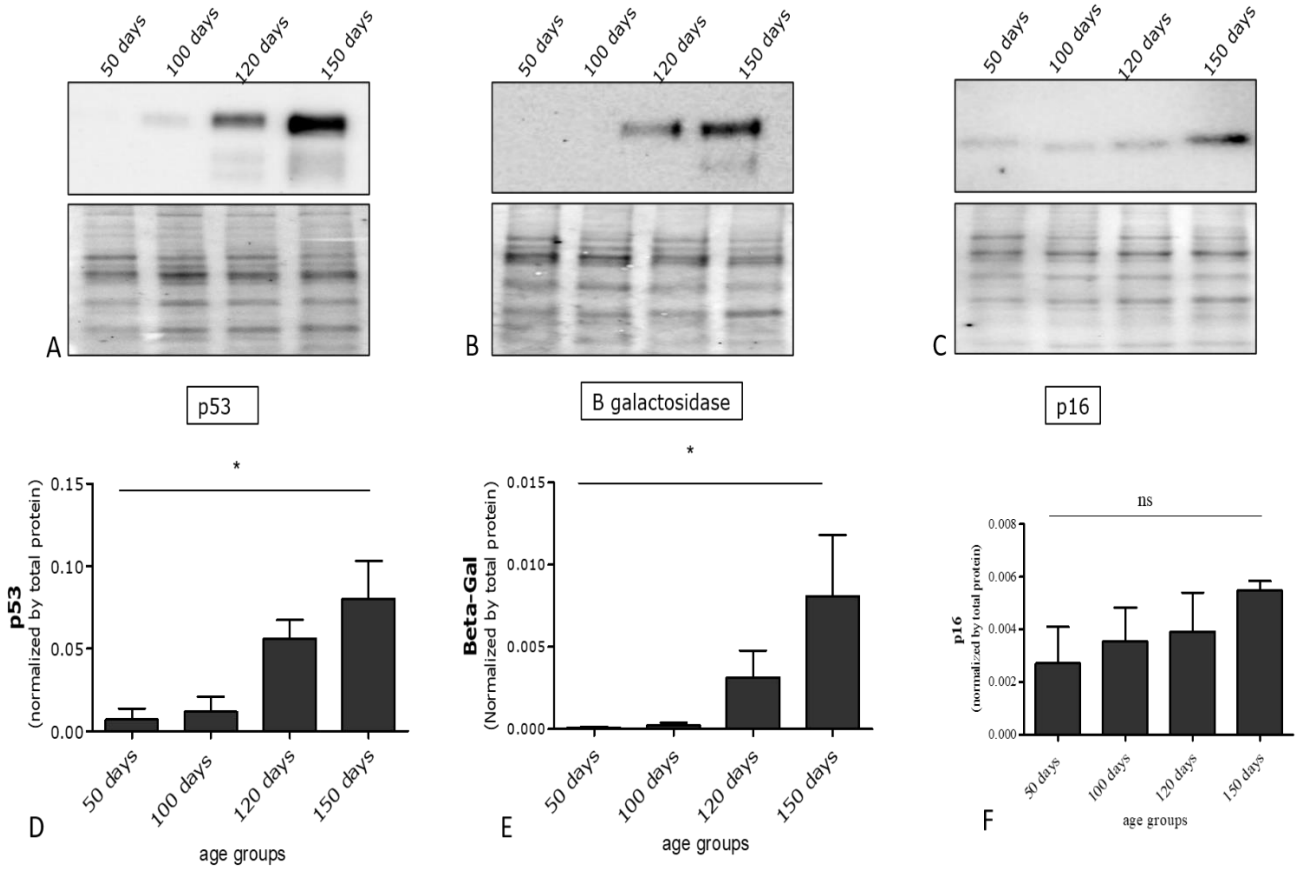

Figure 10. Age-dependent gradual increase of senescence markers in G93A model

The level of p16INK4a, p53, and SA-β-gal proteins in the spinal cord tissue showed a gradual increase measured by Western blot. Quantification of the senescence markers was done by normalizing to the total protein. One-way ANOVA, followed by Bonferroni's Multiple Comparison Post-Test, was performed to establish significant differences among the groups. The asterisk (*) indicates a significant ($P < 0.05$) difference between the youngest (50 days) and the oldest group (150 days) of mice. Each experiment was repeated independently 3 times. Bars represent mean ± SEM, n=3 per group.

2.4.4. CT4 specifically interacts with hSOD1 and the binding increases over-time

The purpose of utilizing a fusion peptide in the current study is twofold. Primarily, to specifically knock down the modified hSOD1 and collaterally, preserve the native ones to perform essential antioxidant activities. The protein Derlin-1, also known as degradation in endoplasmic reticulum protein 1, is part of a protein complex that mediates endoplasmic-reticulum-associated degradation (EARD) and is required to translocate the misfolded/unfolded proteins from ER to cytoplasm for degradation by proteasomal or lysosomal systems. The cytosolic carboxyl-terminal region of Derlin-1 is termed as CT4, composed of 12 amino acids (FLYRWLPSRRGG). The human SOD1 possesses a Derlin Binding Region (DBR), which is concealed in its native conformation. We speculated that conformational changes enforced by various obnoxious stimuli such as oxidative modification could misfold the protein in such a way that unveils the DBR region. With this fundamental notion, CT4 fusion peptide was designed to have three distinct domains: the 11-amino acid transduction domain of HIV TAT protein capable of the crossing cell membrane and the blood-brain barrier, a protein-binding motif that binds explicitly to POIs, and finally, the KEFRQ-related targeting motif destined for lysosomes. In order to establish CT4 and modified hSOD1 binding, in the current study, we have utilized BCL21 E. coli strains for bacterial transformation to grow GST-CT4 proteins. Then the proteins were isolated by bacterial lysis and immobilized by glutathione Sepharose. Next, the G93A transfected N2a cell lysates were incubated with

the glutathione immobilized GST-CT4 protein, which was subsequently centrifuged to separate the pellet and the supernatant and subjected to western blot analysis to detect the CT4 bound misfolded conformation of hSOD1 by utilizing antibody against SOD1. PGEX-4T-1 backbone was used to generated GST protein via bacterial transformation to implicate as a control to compare the binding efficiency of GST-CT4.

Forty-eight hours post-transfection, a mild hSOD1 band was identified at 17 KD locations in the GST-CT4 group, whereas the GST control exhibited no binding to hSOD1. To confirm the CT4 bound SOD1 was the human origin, in the same gel, we ran both the pellets and the supernatant from GST-CT4 and GST groups (figure 2.8). The supernatants contained both mice SOD1 and human SOD1, which could be differentiated in the blot at 15 kDa (lower band) and 17 kDa (upper band) location, respectively, by using total SOD1 antibody (8B10). Forty-eight hours post-transfection, a mild hSOD1 band was identified at 17 KD location in GST-CT4 containing pellets, whereas the GST pellets (control) exhibited no binding to hSOD1. The supernatants showed the mice and the remaining native human SOD1 bands, which did not react with CT4. Next, we experimented with post-1-week G93A transfected cell lysates. The western blot analysis (figure 2.9) revealed a marked increase of CT4 bound hSOD1 bands, indicating the misfolded conformations of G93A SOD1 were augmented over time.

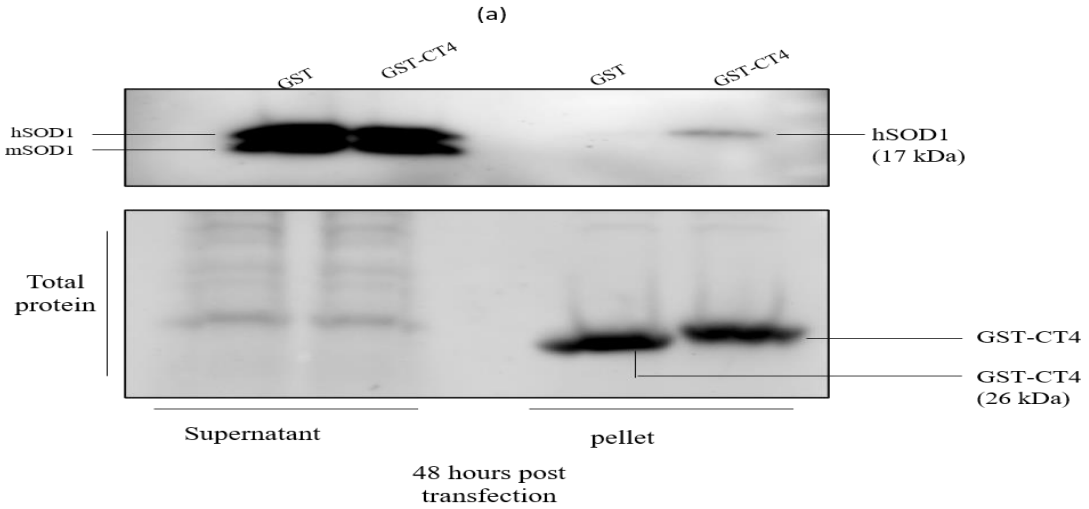

Figure11. CT4 specifically binds to hSOD1.

N2a cell lysates were prepared 48 hours post-transfection with G93A and incubated for 1 hour with GST-CT4, which was immobilized by glutathione. The control group (GST) was devoid of CT4 and similarly treated with G93A containing lysates. The incubation was followed by the separation of the glutathione-GST-CT4 containing pellets and supernatants. The 1st and 2nd lane in the western blot showed the mouse and human SOD1 (8B10 antibody) in the supernatant at 15 kDa (lower band) and 17 kDa (upper band) location. The 3rd lane is the control group without CT4 where the pellets are devoid of any SOD1 binding, whereas in the 4th lane, the GST-CT4 group exhibited CT4 binding with modified hSOD1.

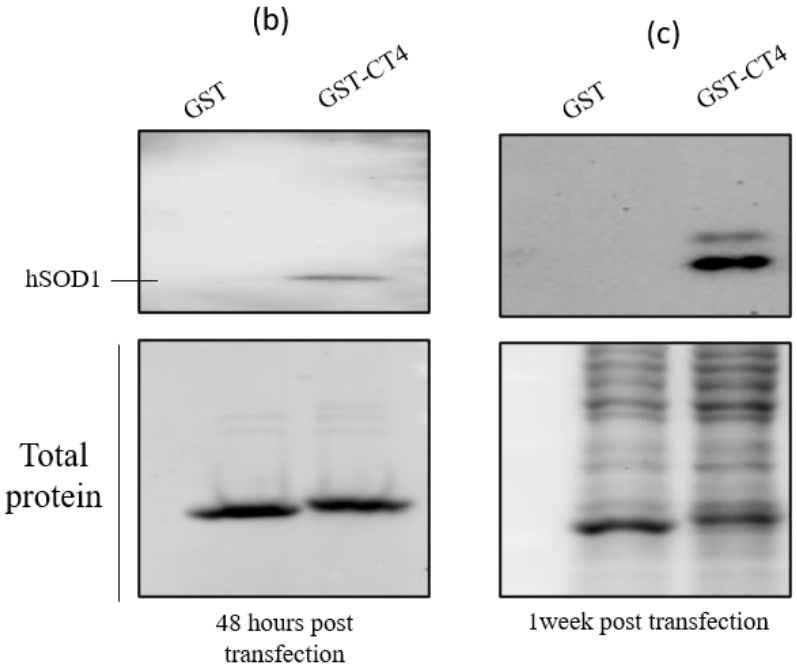

Figure 12. SOD1 -CT4 conjugation was enhanced with time in G93A transfected cells.

Image (b) and (c) showed the binding of GST-CT4 with hSOD1 in G93A transfected N2a cells at 48 hours and 1week post-transfection accordingly. The control group (GST) had no SOD1 binding. Both groups were maintained in G-418 containing medium.

2.4.5. Cellular proliferation is ameliorated by introducing CT4 plasmids into the G93A transfected N2a cells

The earlier findings in the current study depicted that the cellular proliferation declined remarkably at 3rd week in G93A transfected cells. In order to retrieve the proliferation rate, we have re-transfected the N2a cells with GFP tagged CT4 plasmid constructs at the end of two weeks of their initial transfection with G93A. The transfected cells were cultivated in G-418 containing media throughout the experiment. After 1 week of CT4 transfection, the cells were fixed for IF study to assess the proliferation. At the same time, GFP tagged mCT4 was transfected into the G93A group in a separate flask and treated analogously for the equal duration to be utilized as a control group. The result showed that the G93A transfected N2a cells proliferated substantially after CT4 treatment for 1 week, evident by the expression of Ki 67 (red fluorescence signal). [fig. 2.10 (e-h)]. Whereas the G93A group with mCT4 has shown minimal proliferation [fig. 2.10 (a-d)] similar to the earlier findings in (l, o, r) panels of Figure 2.3. The result concluded that the elimination of aberrantly folded SOD1 by CT4 treatment could instigate cellular proliferation.

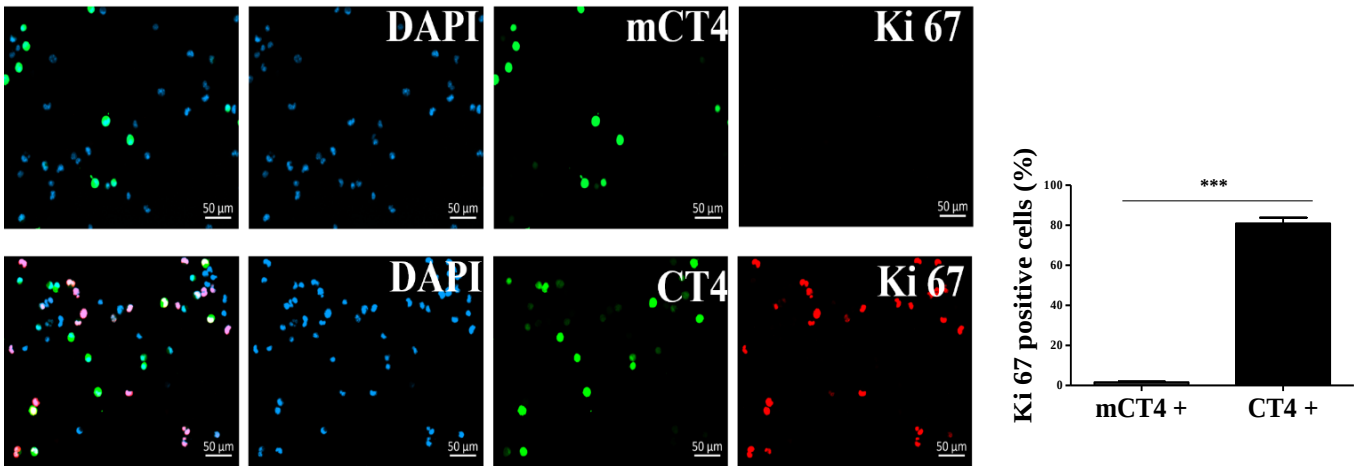

Figure13. CT4 peptide enhanced the (Ki 67) proliferation in G93A transfected N2a cells.

G93A transfected cells at the end of two weeks of their initial transfection were transiently transfected with GFP tagged CT4 plasmid constructs, and an mCT4 transfected group was maintained in a separate

flask to be utilized as control. 1week treatment with CT4 (green signal) significantly increased the Ki 67 expression in the G93A transfected N2a cells (e-g), whereas the control group with mCT4 exhibited no comparable expression of Ki67 (a-d). For statistical analyses, Unpaired t-test was performed to compare the groups. The asterisk (***) indicates a significant ($P < 0.0001$) difference between the groups. Means ± SEM, n=15, n represent images captured from 3 independent cultures. Scale bar=100 µm.

2.4.6. Treatment of the G37R mice at the age of 70 days and 120 days by IP injection (once daily) significantly reduced the oxidatively modified hSOD1.

Two different age groups of animals were treated with TAT-CT4-CTM peptide (20 µg/ kg) by intra-peritoneal IP (intraperitoneal) injection (once daily) consecutively for 1 month beginning at 70 days and 120 days of age. The disease unfolds in the G37R model approximately at 120-125 days. Hence, one group represented mice at their middle age, whereas the latter group was representative of mice at their pre-symptomatic stage. Each of them was compared with their age-matched control group. After 1 month of injection, mice were sacrificed at the age of 100 days and 150 days, and collected spinal cord tissues were prepared and incubated with MalPEG followed by western blot analysis with SOD1 antibody. Both the age groups showed a decline in hSOD1 oxidation after CT4 treatment while the reduction was significant in 100 days old group (figure 2.11 a, b, c) mice compared to their age-matched control group. Assumedly, the hSOD1 oxidation in 150 days old group (figure 2.11 d, e, f) was elevated to a point where the large aggregates were pertinacious to CT4 mediated lysosomal degradation, or this could have resulted from the age-induced deterioration of lysosomal degradation system at the molecular level. However, it revealed that the CT4 treatment could be more advantageous as a preventive therapy to impede the augmentation of large aggregates, which could be water-resistant to break-down.

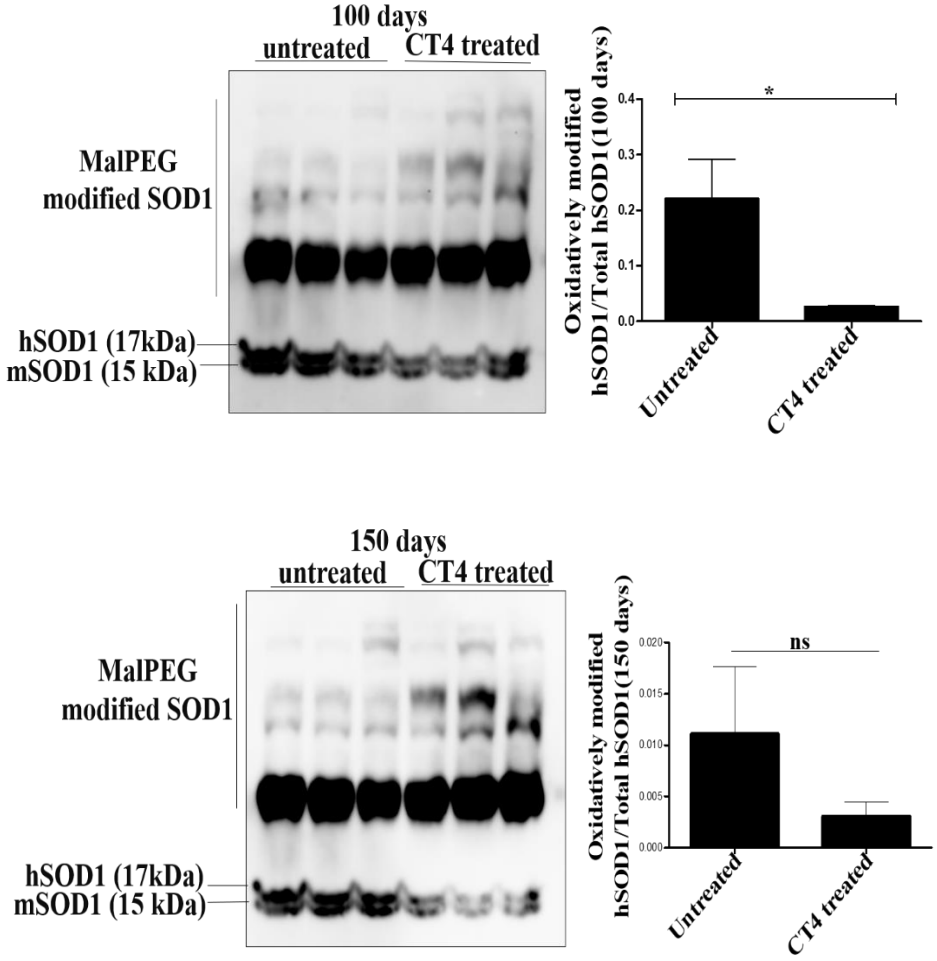

Figure 14. CT4 peptide injection reduced the oxidized hSOD1 in the spinal cord of G37R mice

The CT4 peptide treated (20 µg/ kg) and untreated age-matched control groups were sacrificed according to the protocol and collected spinal cord tissues were incubated with MalPEG with subsequent western blot analysis by using SOD1 antibody (8B10). The result showed that the 1month peptide injection was sufficient to increase the MalPEG modified native hSOD1in both age groups. Parallelly, the oxidatively modified hSOD1 at 17 kDa location is reduced in both 100 (a-c) and 150 (d-f) days group compared to the age-matched untreated group. The mouse SOD1 is identified at 15 kDa location. One-way ANOVA, followed by Bonferroni's Multiple Comparison Post-Test, was performed to establish significant

differences among the groups. The asterisk (*) indicates a significant ($P < 0.05$) difference between the youngest (50 days) and the oldest group (150 days) of mice. Each experiment was repeated independently 3 times. Bars represent mean ± SEM, n=3 mice per group.

2.4.7. knockdown of the aberrant SOD1 conformations by TAT-CT4-CTM fusion peptide treatment in G37R transgenic mouse model reduced the senescence

As mention above, two different age groups, along with their age-matched controls, were treated with a daily injection of the CT4 fusion peptide (20 µg/ kg) for 1 month. Subsequent western blot analysis with the spinal cord tissue samples revealed the expression of senescence markers, which were reduced in synonymously with the oxidized SOD1. Both the age groups 100 days and 150 days old have shown a decrease of SA-β-gal (a-b), p16INK4a [figure 2.12 (c-d), p53(e-f)] markers where the 100 days old group was more responsive to CT4 treatment with a substantial reduction of aging-associated senescence.

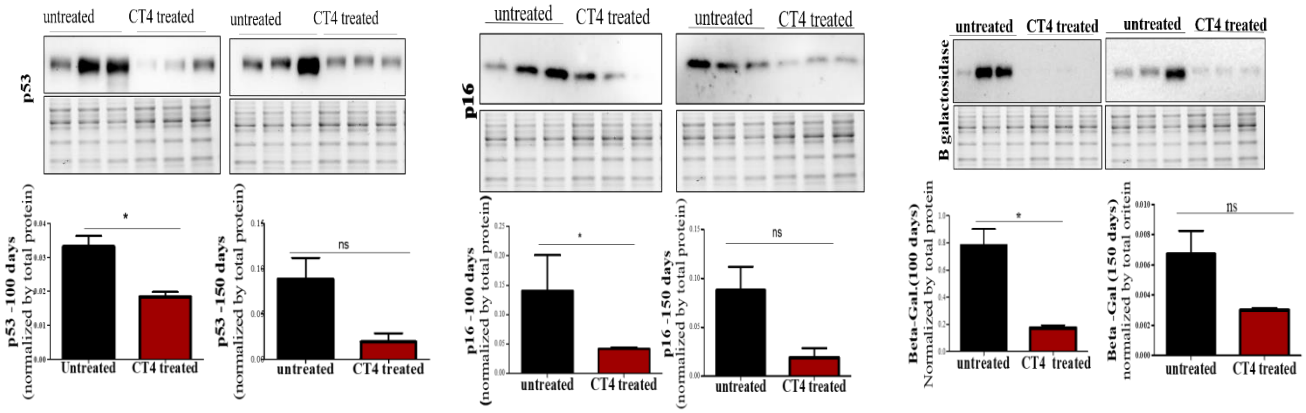

Figure 15. CT4 peptide injection reduced the aging-associated senescence in the spinal cord of G37R mice

The CT4 peptide treated (20 µg/ kg) and untreated age-matched control groups were sacrificed according to the protocol and collected spinal cord tissues were prepared for western blot analysis. The result showed that the 1month peptide injection (once daily) was sufficient to reduce the expression of SA-β-

gal (a-b), p16INK4a (c-d), p53(e-f) where the 100 days old group have shown a significant reduction of senescence in response to CT4 treatment. One-way ANOVA, followed by Bonferroni's Multiple Comparison Post-Test, was performed to establish significant differences among the groups. The asterisk (*) indicates a significant ($P < 0.05$) difference between the youngest (50 days) and the oldest group (150 days) of mice. Each experiment was repeated independently 3 times. Bars represent mean ± SEM, n=3 mice per group.

2.5. Discussion

The current study has addressed the mechanistic relevance of oxidatively modified SOD1 to pathological aging utilizing the ALS model as an experimental tool. Earlier studies have demonstrated the aging-associated accumulation of oxidized proteins(156). However, there are fewer examples that directly pinpointed their contribution to accelerating aging by evaluating aging-associated markers. For instance, accelerated aging induced by carbonylated protein content is a pathological phenomenon associated with HIV infection. The hypothesis was established by correlating protein oxidation and markers of oxidative DNA damage. In the current study, we have observed an age-dependent rise of oxidized SOD1 protein in the G93A mouse model. The G93A is a mutant human SOD1, harboring a single amino acid substitution of glycine to alanine at codon 93. Previously, it was confirmed that the G93A and wild variety of SOD1 orchestrates similar pathological consequences when they encounter toxic gain of function by oxidative modification (96). Nevertheless, compared to wild type SOD1, G93A is extensively studied due to its aggressiveness, conspicuous pathological events(77). The toxicity of G93A is also indicated by our in-vitro study on N2a cells in figure 2.3, 2.4, and 2.5, where G93A transfected cells approached senescence vigorously compared to the wild type one. The in vitro data validated the G93A model in the current pursuit of study.

Previous data from our lab has demonstrated the age-dependent rise of oxidized SOD1 aggregates in the G37R model when compared among three different age groups(29). Furthermore, transgenic mice harboring the wild type human SOD1 gene have also exhibited escalation of oxidatively modified SOD1 in 450 days old group compared against 210 days old mice(94). We extended our experiment by including four different age groups G93A mice, the average life span of which is 157.1^{+}/- 9 days, and the disease onset occurs at around 130 days of age (https://www.jax.org/). In this context, the four age groups in the current study included young disease-free mice (showing no symptoms), mice at pre-symptomatic and symptomatic stages (100 & 120 days) to older mice reaching their endpoints (150 days). Incubation with MalPEG showed that the oxidatively modified hSOD1 progressively increased with age and exhibited a significant escalation close to their endpoint compared to the youngest age group (figure 2.6). The earlier study showed that MalPEG modification was human SOD1 specific, and the cysteine 111 group was their initial target for oxidation and subsequent aggregation(29). In agreement with that, the current findings further emphasized the cysteine 111 mediated and aging-related oxidation of SOD1 established by decreased accessibility to MalPEG in the western blot. The G93A transgenic mice are widely recognized for their short life span, yet no other study has experimentally explored molecular influences. However, modified SOD1 was shown to negatively impact the transport function across the mitochondria (162) and endoplasmic reticulum (101) and mRNA stability (163) and the proteasomal activity across the cytoplasm, which collectively interrupted the cellular homeostasis, and contributed to the rise of oxidative stress that could assumedly potentiate the aging process. Our study for the first time has revealed the synchronous and comparable rise of oxidized SOD1 and senescence markers of aging in the G93A model. Until now, much of the fundamental understanding of cellular senescence has been built on the study of mitotic cells, considering the primary characterization of senescence cells as a stable growth arrest. Nevertheless, the most recent findings suggest that post-mitotic cells like neurons from different regions of the mouse brain can exhibit the typical features of senescence in response to damage

accumulation from aging or injury. For example, p53 and its downstream product p21 exert senescence in mitotic cells by arresting cell cycle and producing reactive oxygen species (ROS). Similarly, p53 exerts neuronal senescence by generating ROS production without the involvement of the cell cycle(59). In this context, senescent cells in the current study could be the glial cells, endothelial cells, stem cells, and neurons as well. In fact, the higher expression of hSOD1 in motor neurons of G93A transgenic mice makes it more vulnerable to stress-induced senescence and aging(164).

To validate our hypothesis, we specifically knocked down the modified hSOD1 by CT4 plasmid constructs and CT4 fusion peptides. Relevant studies on ALS have detected a 30% reduction in mean, median, and maximum life span in $SOD1^{-/-}$ mice(165), which showed severe distal axonopathy, dented muscle mass, and elevated oxidative stress(166). Apart from these phenotypic accelerations, $SOD1^{-/-}$ mice disported cellular aging by expressing a higher level of senescence markers(167). Knockdown of SOD1, achieved previously by targeting the SOD1 gene or SOD1 mRNA (168)(169), has been shown to effectively slow down disease progression and prolong survival in mouse models of ALS. These strategies did not target the post-translationally modified versions of SOD1 and thus may not eventually develop into a cure for ALS. Besides, manipulating gene expression at the DNA level is currently inapplicable to human beings, and RNA interference is known to have off-target effects that might affect other innocent proteins (170). To overcome such hassles, adopting a simple, non-virally mediated, membrane-permeable peptide-based system that selectively knock-down the aberrant SOD1 conformations would be a more reasonable and approachable strategy to reveal the underpinning mechanisms associated with pathological aging. The in vitro study with G93A transfected N2a cells showed a significant rise in the proliferation rate after the 1-week CT4 treatment. The in vivo study reflected similar findings where a 1month IP injection of CT4 fusion peptide to G37R mice reduced the oxidized hSOD1 and the senescence markers in the spinal cord tissues. We have experimented on two different age groups, which were 70 days and 120 days old at the beginning of treatment. Compared to

the older group, the younger one exhibited an enhanced response to CT4 treatment by showing a potential decline in SOD1 modification (figure 2.11) and the senescence markers mentioned earlier (figure 2.12). Assumedly, the elevation of oxidized hSOD1 in 150 days old group reached a critical point where the large aggregates were pertinacious to CT4 mediated lysosomal degradation, or this could have resulted from the age-induced deterioration of lysosomal degradation system. However, the findings revealed that the CT4 treatment could be more advantageous as a preventive therapy to impede the augmentation of aberrant hSOD1, which could later form stubborn aggregates, resistant to degradation.

Our results have shown that post-translational oxidation of SOD1 coincides with the increase of senescence markers, and selective degradation of modified SOD1 by CT4 peptide could reduce the aging-associated senescence in ALS mouse models. The current study, for the first time, has established that post-translational oxidation of SOD1 is a mechanistic fomenter of cellular senescence and aging in ALS. Besides, the proposed fusion peptide offers a therapeutic remedy against early death from ALS.

Chapter 3: Conclusion, Limitation, and future direction

Aging is an undeniable phenomenon, yet how we age may vary within individuals. Pathological aging is an accelerated form that intensifies the sufferings and ensures early demise. Considering a disturbingly short life span in ALS patients, the current study has identified a mechanistic cue to the pathological aging in ALS patients. SOD1, one of our primary antioxidant enzymes, is well-known to be associated with both familial and sporadic ALS through toxic gain of function. Earlier works have shown the vulnerability of cysteine 111 residue in SOD1 to oxidative post-translational modifications, which subsequently give rise to toxic conformations of SOD1. Our result identified that oxidized SOD1 could instigate cellular damage up to a critical limit where the cells become senescent. Senescence is one of the commonly used biological measures of aging, which involves substantial molecular and organelle damages. To further confirm our results, we could also focus on different molecular pathways mentioned in chapter 1.3 and susceptibility of the individual organelle; instead, we focused more on the downstream consequence of the aging process like senescence. However, we have confirmed the hypothesis by utilizing CT4 fusion peptide, which binds to the DBR (Derlin Binding Region) of misfolded SOD1. DBR is a small segment; it is relevant to expect that oxidative modification might misfold the SOD1 proteins into non-native conformations, which expose the DBR, even though the global structure is not the same. Whether CT4 could remove all the toxic SOD1 conformations, is still confusing. For instance, the SOD1, which are only oxidized yet not misfolded or larger aggregates concealing DBR regions, may exclude the CT4 mediated degradation. In this regard, the functional efficiency of lysosomes and associated effectors of the autophagy lysosomal pathway are also significant variables. Again, a study, including a larger number of animals, could have helped an extensive analysis.

The study could have significant implications in translational research. Oxidative modification of SOD1 could be assessed as a biomarker of pathological aging in ALS by utilizing available patient samples like serum or CSF. In this regard, we are currently approaching the ELISA technique to precisely measure the oxidized SOD1 by using Dimedone, which mainly binds with cysteine 111 oxidized SOD1.

Dimedone, when binds to oxidized SOD1, is detected by the anti-dimedone antibody. Considering the implication of SOD1 PTM in multiple age-related diseases, adopting CT4 fusion peptide as a potential interventional strategy in global aging, could be a reasonable attempt shortly.

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