Mending the broken in amyotrophic lateral sclerosis: DNA damage and repair in motor neuron degeneration

Byung Woo Kim, Lee J. Martin*

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease that causes paralysis and respiratory failure (Petrov et al., 2017). The driving mechanisms are unknown, and there are no effective treatments (Petrov et al., 2017). Aging and a few gene mutations, a common one being missense mutations in superoxide dismutase-1 (*SOD1*), are risk factors for ALS (**Figure 1**). The recent Food and Drug Administration approval of edaravone for the treatment of ALS putatively supports a role for oxidative and nitrative stresses in the disease processes (**Figure 1A**). DNA damage, abnormalities in DNA repair, and other nuclear abnormalities are implicated also in the pathogenesis of human ALS (Bradley and Krasin, 1982; Kim et al., 2020). DNA damage as an upstream pathogenic event in human ALS is supported by evidence for p53 activation and its import into the nucleus of motor neurons (Martin, 2000), and hyperactivation and nuclear accumulation of apurinic/apyrimidinic endodeoxyribonuclease-1 (Shaikh and Martin, 2002). Kim et al. (2020) discovered that upper and lower motor neurons in postmortem central nervous system (CNS) from ALS patients, mostly sporadic ALS in comparison to age-matched controls, accumulate several different types of DNA lesions and engage a prominent DNA damage response (DDR), as evidenced by accumulation of nuclear Abelson non-receptor tyrosine kinase and breast cancer type 1 susceptibility protein, and the serine/threonine protein kinase ataxia telangiectasia mutated activation (**Figure 1A**). Apyriminidinic sites, single-stranded DNA, oxidized DNA, and DDR proteins are present in motor neurons at pre-attritional stages and throughout the somatodendritic attritional stages of neurodegeneration (Kim et al., 2020). Motor neurons with DNA damage are also positive for activated p53 and cleaved caspase-3 (**Figure 1A**). These recent findings support the concept that, in human ALS, the motor neuron degeneration is a cell-autonomous form of programmed cell death (Martin, 1999).

It is a major challenge interrogating this hypothesis in clinically relevant disease-affected living cells. Animal models and animal cell systems may not reflect accurately the human disease state (Petrov et al., 2017; Martin and Chang, 2018). Human induced pluripotent stem cell (iPSC)-derived motor neurons with familial ALS-causing gene mutations can be used to directly test the hypothesis that ALS motor neurons accumulate genomic DNA lesions and engage DDR as part of the neurodegenerative process and, possibly, antecedent to neuronal cell death (**Figure 1A**).

Recent work challenges the long held assumptions that experimental mammalian animal modeling of nervous system injury and disease is directly reflective of, and relevant to, human neuron biology and should be a launchpad for human CNS disease- and injury-related clinical trials (Petrov et al., 2017; Martin and Chang, 2018). Neuronal cell culture studies have revealed that DDR, DNA repair, and cell death mechanisms are different in human neurons compared to mouse and pig neurons; moreover, fundamental differences in promoter CpG island DNA methylation patterns exist between human neurons and mouse neurons (Martin and Chang, 2018). Importantly, the promoter CpG methylation of gene networks involved in the unfolded protein response (UPR) and proteasome biology and genes involved in DNA repair are different in stressed human and mouse neurons (Martin and Chang, 2018). Intriguingly, pig neurons, derived from olfactory bulb neural stem cells, have DDR and DNA repair more closely like human neurons than mouse neurons (Martin and Chang, 2018). Uniquely hominid neuronal cell injury response, DDR, proteasome regulation, and cell death mechanisms would be paradigm shifting for experimental neuropathology and the modeling of human CNS injury and disease. Studies of patient *in vivo* imaging, plasma and cerebrospinal fluid biomarkers, postmortem human CNS, particularly studies of early disease events, should be encouraged. However, study of human postmortem CNS yields static data that is often interpreted as temporal sequences of events regarding mechanisms of disease, but this is not a true dynamic representation of disease. Human iPSC-derived motor neuron cell culture models are critical to study mechanisms of disease and therapeutics in living ALS and control motor neurons.

iPSCs are a type of cell that originates from an adult cell, such as a skin fibroblast or a peripheral blood mononuclear cell, that is experimentally genetically reprogrammed using specific genes (*Oct4*, *Sox2*, *Nanog*, and *Lin28*) introduced by genome-integrating and –nonintegrating methods or direct protein transfer (Takahashi et al., 2007). A defining feature is that iPSCs can generate the three primary germ layers, as if it were an embryonic stem cell, but it is not derived from human embryos (Takahashi et al., 2007). For studying disease mechanisms, human iPSCs can be generated from patients with disease, such as ALS, and are called patient-derived iPSCs. In the study by Kim et al. (2020), an ALS patient-derived iPSC line expressing the SOD1-A4V mutation was used. In addition, normal human cells that have been genome-edited to introduce disease-causing gene mutations can be used. An advantage of using genome-editing methods, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), is that isogenic control cells with the identical genetic background, except for the specific gene mutation, can be prepared.

Kim et al. (2020) introduced a SOD1-G93A missense mutation by CRISPR-Cas9 genome editing technology into a healthy control iPSC line. CRISPR-Cas9-mediated homology-directed DNA repair is a prime method for gene editing of human cells. The technology is complex, but, briefly, Cas9 nuclease, guide RNA, and specific single-stranded DNA donor oligonucleotide template that are co-electroporated into iPSCs. The guide RNA has a sequence that recognizes the target DNA-gene region of interest for navigating the Cas9 to that locus to cleave the double-stranded DNA as refined by the protospacer adjacent motif. The exogenous donor DNA, through sequence homology, is used by homology-directed DNA repair to repair the DNA break and introduce the missense point mutation. The genome editing of cell clones is confirmed by DNA Sanger sequencing (Kim et al., 2020). Challenging obstacles of the CRISPR-Cas9 technique are low efficiency of homology-directed DNA repair, genomic rearrangements, and off-target effects. Gene candidates potentially vulnerable to off-target effects can be identified based on a COSMID web tool (Kim et al., 2020).

For ALS cell models, critically characterizing the differentiated neuronal system as faithfully representing motor neurons is paramount in importance (**Figure 1B**). The gold standard is the human lower motor neuron in brainstem and spinal cord with their distinctive morphological signatures (**Figure 1B**). Most iPSC-derived motor neuron studies have relied on staining for *motor neuron and pancreas homeobox-1*, also called Hb9, transcription factor and the cholinergic enzyme choline acetyltransferase. Hb9 is useful because it is believed to be required for the consolidation of motor neuron identity; however, a sizable pool of spinal cord interneurons also expresses Hb9 (Chang and Martin, 2011). When using choline acetyltransferase as a marker, it is often overlooked that spinal cord sympathetic and parasympathetic preganglionic neurons, peri-central canal interneurons, and some dorsal horn neurons are all choline acetyltransferase-positive. Generally, these cells are not vulnerable in ALS. Moreover, within the somatic motor neuron echelon, the cells of interest in ALS, there are α, β, and γ motor neurons that have dissimilar vulnerabilities in ALS. Morphology, seen clearly by the cytoskeletal proteins microtubule-associated protein-2 and β-tubulin III, has considerable merit in classifying motor neurons in cell culture (**Figure 1C**–**F**) because of the large and multipolar soma that mimics faithfully human motor neurons *in vivo* (**Figure 1B**).

Because ALS motor neurons showed significant DNA damage accumulation, DDR, and promoter hypomethylation in DNA repair

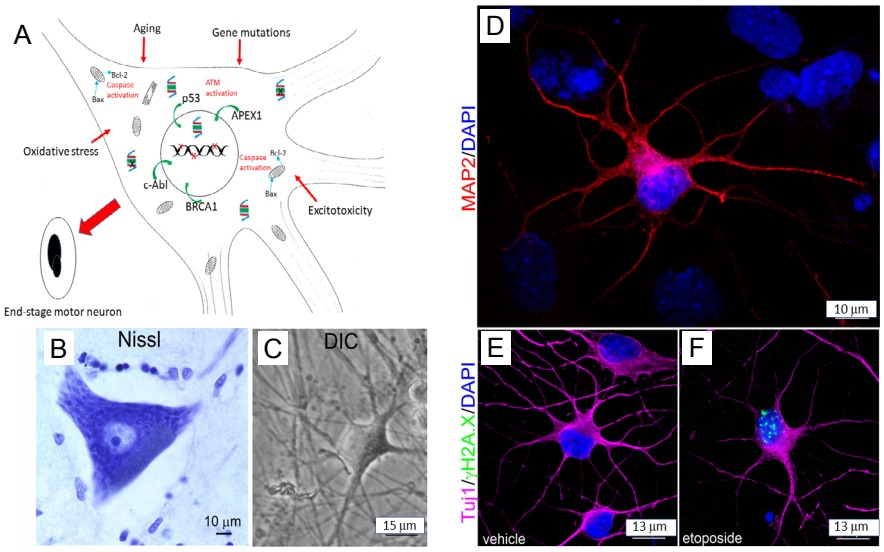

Figure 1 | **Human motor neurons in amyotrophic lateral sclerosis (ALS).**
(A) Diagram of a motor neuron with organelles, including cytoskeleton, mitochondria, endoplasmic reticulum, and proteasome (tailed barrel), and the putative mechanisms of motor neuron degeneration in human ALS. DNA damage or some form of genome instability (X in nucleus) is upstream, possibility triggered by aging, abnormal mutant proteins consequent to gene mutations, proteasome compromise (X in cytoplasm), excitotoxicity, or oxidative stress. The ability of motor neurons to mount a DNA damage response (DDR) is robust, but if DNA repair is incomplete, lacks fidelity, or is not sustainable motor neurons engage a program of attrition and death (lower left profile). (B, C) Classification of human induced pluripotent stem cell (iPSC)-derived neurons as motor neurons in reference to the gold standard motor neuron found in human spinal cord (B) shown here as an α-motor neuron present in the lateral cell group (group IX) of a control human lumbar spinal cord (B). (C) A live human iPSC-derived neuron, seen by differential interference contrast (DIC) microscopy, cultured for 30-days post-differentiation exhibiting a large cell body and a multipolar morphology consistent with a classification of motor neuron. (D) Immunofluorescent immunophenotyping of human iPSC-derived neurons for cytoskeletal protein microtubule-associated protein-2 (MAP) revealing the large soma and multipolar dendrites and the 4′,6-diamidino-2-phenylindole (DAPI) nuclear staining (blue) to show the large nucleus with dispersed chromatin, all features consistent with a motor neuron classification. (E, F) Immunofluorescent immunophenotyping of DNA damage in human iPSC-derived motor neurons at 30-days post-differentiation. Motor neuron morphology was visualized by β-tubulin III antibody (Tuj1), and DNA double-strand breaks in etoposide-treated cells were detected by γH2A.X-postive foci. In vehicle-treated control human motor neurons, no DNA double-strand breaks were detected in the nucleus (blue), while in etoposide-treated motor neurons DNA double-strand break foci were prominent in the nucleus.

genes in human postmortem CNS tissues (Kim et al., 2020), and realizing that these are all static assessments, an investigation of DNA repair capacity in living human iPSC-derived motor neurons was needed (**Figure 1E** and **F**). iPSC-derived motor neurons were challenged with the topoisomerase-II inhibitor etoposide to induce DNA double-strand breaks. DNA damage accumulation was striking in the nucleus of motor neurons treated with etoposide (**Figure 1E**), but not in vehicle-treated motor neurons (**Figure 1F**) visualized by γH2A.X immunoreactivity, a serine-139 phosphorylated form of H2A and an established marker for DNA damage. Steady-state repair of baseline endogenous DNA double-strand breaks in ALS motor neurons was like control at 30 days of culture (Kim et al., 2020). After 1 hour of 10 μM etoposide exposure, nearly all motor neurons in control and ALS cultures accumulated similar levels of DNA damage as seen by the accumulation of γH2A.X foci (**Figure 1F**). Repair of DNA damage, as seen by disappearance of γH2A. X foci, was similar in SOD1 mutants and control at all recovery periods (Kim et al., 2020). Thus, iPSC-derived motor neurons with SOD1 mutations responded to DNA damage and repaired DNA damage with kinetics like control motor neurons.

In summary, Kim et al. (2020) found in postmortem CNS tissue evidence for the accumulation of several different forms of DNA damage and engagement of a significant DDR in human ALS motor neurons demonstrated by activation and nuclear recruitment of DNA damage sensor proteins (**Figure 1A**) and DNA repair gene hypomethylation. These results were complemented by evidence that human ALS iPSC-derived motor neurons can engage a strong DDR with a repair capacity like wildtype motor neurons. An important caveat to recognize though is that this iPSC work is currently representative of SOD1-related familial ALS, and this is small section of the total ALS burden. Notwithstanding, the fidelity of the DNA repair and whether it is sustainable in ALS motor neurons at different ages needs further study.

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