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Chapter 4

SOD1, from Bench to Bed: New Role for the Oldest Protein Implicated in ALS

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Abstract

In 1993, the first superoxide dismutase 1 (SOD1) mutation in amyotrophic lateral sclerosis (ALS) patients has been described by Rosen et al. successively, the scientific literature focused on the role of SOD1 in the pathogenesis of ALS. While a clear genetic scenario has been presented, heterogeneous data have been formulated regarding transcriptional and post-transcriptional regulation of SOD1 so far. In particular, the dilemma concerns the SOD1 protein expression, in the direction of a loss of function of the wild-type SOD1 or a toxic gain of function of the altered SOD1, both in FALS (mutant-SOD1) and in SALS (misfolded-SOD1). In this chapter, we focus on the evolution of scientific knowledge about SOD1 protein in ALS patients, reviewing in detail the results obtained using peripheral blood cells in this research field. To conclude, we propose a brief summary of the described clinical correlation and discuss the possible SOD1 implication as a biomarker of ALS.

Keywords: trascriptional regulation, post-trascriptional regulation, amyotrophic lateral sclerosis (ALS), superoxide dismutase 1 (SOD1), biomarkers, protein aggregation

1. Introduction

Cu/Zn superoxide dismutase (SOD1) is a 32-KDa homodimeric enzyme that assumes a pivotal role in the scavenging process of the toxic superoxide radicals in the cells [1]. SOD1 is highly expressed in the cytoplasm, but in the 1980s, Chang and colleagues identified SOD1 in other sub-cellular compartments, such as nucleus, lysosomes, and mitochondria [2]. In the 1990s, the scientific literature focused on the genetic and biochemical characterization of SOD1, and in

1993, Rosen et al. [3] described the first SOD1 gene mutation associated with amyotrophic lateral sclerosis (ALS) [3]. ALS is an adult-onset neurodegenerative disease that affects selectively cortical, bulbar, and spinal motor neurons, leading invariably to a fatal prognosis. The majority of ALS cases (90–95%) are sporadic (SALS), whereas 5–10% are familial (FALS), with genetic mutations dominantly inherited. Among these, more than 150 SOD1 mutations have been reported that are responsible for 20% of FALS, but also 1% of SALS [4]. During the last 20 years, many studies were focused on SOD1 protein in the pathogenesis of ALS, both for FALS and for SALS. Hitherto, some aspects remain unclear: (1) SOD1 alterations give selective loss of motor neurons, even though the enzyme is ubiquitous in every cell; (2) mutations confer SOD1 enzyme's loss of function reducing antioxidant activity but, in some way, mutated enzyme can acquire toxicity; (3) the ALS phenotype doesn't reflect the common presentation of the loss-of-function-related diseases, in terms of age of onset, clinical spreading, and evolution. In this chapter, we would like to pass through the recent understandings about SOD1 protein in the pathogenesis of ALS, in particular focusing on its aggregation in peripheral blood cells and its transcriptional/post-transcriptional regulation.

2. SOD1 and ALS models

2.1. Investigating SOD1-mediated pathogenesis in different cellular models

The SOD1 gene is located on chromosome 21q22.11 (**Figure 1**), and more than 150 mutations have been discovered since 1993, all of them proving to be causative of ALS.

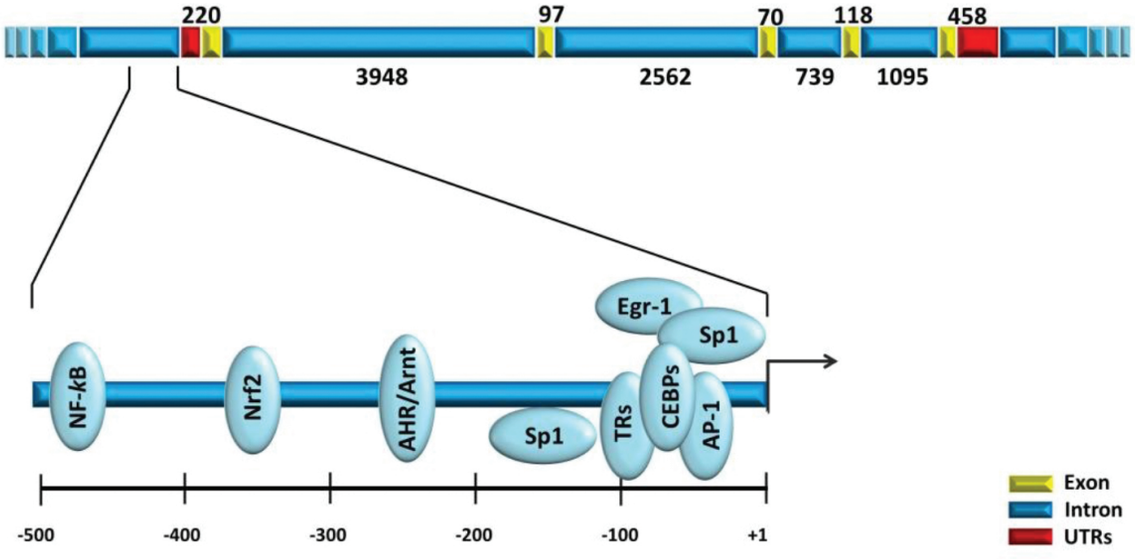

Figure 1. Structure of SOD1 gene.

The discovery of SOD1 mutations in ALS patients allowed the possibility to create transgenic models of the pathology. As familial and sporadic forms of ALS demonstrated to be clinically

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undistinguishable, in the 1990s SOD1-G93A mouse model was realized and allowed for an in-depth stepwise exploration of the neurodegenerative process occurring in ALS.

Conversely, a different scientific approach applies to the understanding of the mechanisms of disease directly in humans. The major limitation is represented indeed by the scarce availability of samples in sufficient quality and high relevance for ALS [5], being spinal cord, brain stem, motor cortex, and muscles as the primary targets of the disease process. The first criticality towards the use of post-mortem tissues is that they represent the end stage of the disease. In second place, the quality of tissues may be compromised by the interval after death and protein alterations occurring independently of ALS that may affect the specificity of the finding [6]. Thus, the proximity of the tissue to the disease pathogenic process, and thus the possibility to uncover a biologically meaningful, highly specific, marker of disease progression, is at the expenses of the accessibility of the given tissues and the opportunity to sample them more than once.

Regarding muscle biopsy, one of the advantages is the shorter processing time compared to post-mortem tissues, but at present there are no sufficient data to support this method of analysis. The presence of neurogenic atrophy is peculiar, but non-disease specific, of ALS. Besides that, these alterations are known to occur late during disease course and they could be measured less invasively with electrodiagnostic testing [6, 7]. As a last consideration, most of the changes observed at the muscle sample are not acknowledged to belong to some specific biochemical pathways or to be just a mere consequence of the denervation process [7, 8].

Because of the large feasibility for repeated testing and the presence of standard operating procedures, blood and cerebrospinal fluid (CSF) have become the most popular biofluids deployed for ALS research [7]. Indeed, these features allow for longitudinal sample collection at high quality and minimal inter-assay differences. On the contrary, both matrices will contain only diluted concentrations of the protein of interest, which can turn into an advantage if the disease process for which the biomarker is released occurs at multiple scattered points within central nervous system (CNS) impossible to be sampled [5]. Furthermore, there is a massive dynamic range of proteins within these two fluids that is present at several orders of magnitude above the candidate biomarkers and could interfere with their detection. CSF is the closest of the two to the CNS compartment, and thus it is more likely to reflect pathological processes occurring within it; in contrast, blood-brain barrier is narrowing the amount of biomarkers possibly circulating in serum or plasma, even though recent studies revealed an impaired function of the latter [9]. On the clinical ground, lumbar puncture is definitely more invasive than venopuncture and subject to an increasing number of variables, such as the possibility of contamination with blood at the side of the puncture and the heterogeneity of sampled volume. Serum or plasma collection is much more accessible, and patients are more compliant to multiple sampling [10]. Both these biofluids can be influenced by multiple systemic phenomena at the time, such as inflammation, dysmetabolism, or damage at the neuromuscular level, which could increase the sensitivity and also act as confounding factors in the interpretation of the source of these candidate biomarkers [5].

Considering the ubiquitous nature of SOD1, and the aforementioned privilege of multiple blood sampling for the research of this highly progressive, severely disabling disease, SOD1-

mediated pathogenesis of ALS has been extensively investigated through the use of blood cells. Moreover, damaged metabolism of lymphocytes was studied in other neurodegenerative diseases, confirming the pertinence of these cells as an appealing tool in understanding the pathogenesis of neurodegeneration [11].

2.2. SOD1 aggregation

In the 1990s, early studies on erythrocytes and lymphocytes of mutated SOD1 FALS proved decreased concentrations and altered the activity of the enzyme years before the onset of pathology [12–14]. However, this modification was restricted to mutated SOD1 ALS patients. In the last years, a growing body of studies showed peripheral blood mononuclear cells (PBMC) of ALS patients and, regardless of the genetic basis, displayed distinctive traits compared to healthy and neurological controls. These alterations mainly concern an aberrant antioxidant response reflected by mitochondrial and calcium-dysregulated metabolism [15] and decreased Bcl-2 and SOD1 expression in peripheral lymphocytes [16, 17] accompanied by the inability to down-regulate these proteins under oxidative stress [16].

In 2010, while investigating mRNA SOD1 expression in lymphocytes from SALS patients as a potential explanation for the decreased levels of SOD1 protein, Gagliardi et al. [18] found that there is increased transcription of SOD1 in the lymphocytes of SALS (compared to healthy and neurological controls). Similar enhanced expression of SOD1 mRNA was found in selected sections of motor spinal cord and brain stem of ALS patients as well, though this phenomenon was absent from cerebellum and non-motor areas of the same patients. At the same time, SOD1 protein levels in the disease-affected tissues from SALS patients did not show any statistical difference compared to controls [18]. These observations imply that SOD1 increased transcription and the decreased protein expressions are disease-specific cellular events, which are apparent, and thus possible to study, at peripheral level. This gap of knowledge between mRNA and protein levels of SOD1 in ALS patients opened a new scenario, in which the study of sub-cellular compartments was pivotal. Initially, a conformational modified SOD1 was observed both in SALS and FALS (misfolded SOD1 and mutant SOD1 respectively), instead of wild-type SOD1 [19]. The pathogenic role of misfolded SOD1 was first hypothesized by Gruzman et al. [20], and additional evidence was subsequently provided by other authors [21, 22]. Specifically, Forsberg et al. [22–23] found similar misfolded SOD1-Bcl2 aggregations in SALS and Pasinelli et al. [24] described it in FALS (mutant SOD1-Bcl2) [23, 24]. Given that, the hypothesis was that wild-type SOD1, under many environmental triggers, became misfolded SOD1 with reduced antioxidant activity (loss of function) and acquired toxic property as mutant SOD1 has [25]. Cereda and co-workers [26] demonstrated a bigger total amount of SOD1 in PBMCs of SALS patients than in cells of healthy controls or Alzheimer patients [26]. Curiously, the SOD1 distribution allowed to distinguish two different categories of patients: the first group had perinuclear SOD1 aggregates and the second one higher nuclear SOD1. In addition, Cereda [26] observed that patients with more perinuclear SOD1 expressed a major amount of insoluble proteins while the other group, characterized by predominant nuclear SOD1, had especially soluble fractions. Eventually, the authors tested whether these findings had clinical correlates (see **Table 1** for general characteristics of patient cohort). Indeed, longer

disease durations from onset to time of sampling and from onset to last visit were found in patients with larger amount of soluble nuclear SOD1 (**Figure 2**, **A**, **B**, $p < 0.05$). In contrast, no significant correlation was observed between nuclear SOD1 ratio and duration from time of sampling to last visit (data not shown). At the same time, a mild increasing trend was found between patients with high nuclear SOD1 and early ALS stage as assessed by ALSFRS-r score at sampling (**Figure 2C**, $p > 0.05$). If these data were confirmed in larger cohorts followed-up longitudinally and employing specific antibodies against misfolded SOD1, this hallmark could be a useful biomarker for stratifying patients according to the pace of progression of the disease.

Clinimetrics	Patients
Gender	7/12
Female/male	
Age of onset (years)	61.2 ± 2.75
Mean ± SEM	
Diagnostic latency (months)	14.8 ± 4.38
Mean ± SEM	
Site of onset	9/10
Bulbar/limb	
Disease duration at sampling (months)	25.4 ± 7.26
Mean ± SEM	
ALS-FRS_r at visit	38.7 ± 2.11
Mean ± SEM	
Progression rate at baseline (PRB)	0.55 ± 0.23
Mean ± SEM	
Disease duration from baseline to last visit/death (months)	50.2 ± 11.18
Mean ± SEM	
UMN/LMN dominance	1/1/6/6/5
Exclusive UMN/dominant UMN/both UMN-LMN/dominant LMN/exclusive LMN	
Cognitive involvement	3/16
Yes/no	
Respiratory involvement	11/8
Yes/no	
SOD1 localization	13/6
High nuclear fraction/low nuclear fraction	

PRB, progression rate at baseline that is calculated as (48-ALSFRS-r score at baseline)/timeline between onset of disease and baseline visit; PRB values <0.5 between 0.5–1 and >1 imply patients are slow, intermediate, or fast progressors, respectively.

Table 1. Characteristics of patients.

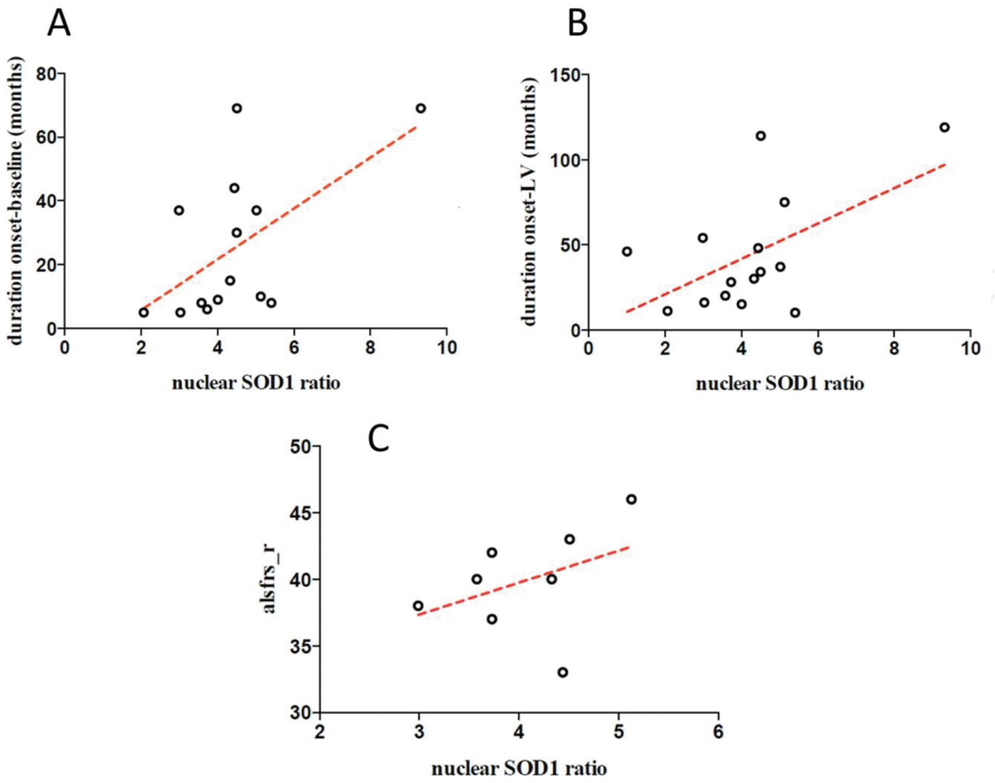

Figure 2. Correlations between nuclear SOD1 and clinimetrics.

Furthermore, this mechanism of pathogenesis could be present in other neurodegenerative disease (such as Parkinson's disease, Alzheimer dementia) and in different conditions, such as prion disease or amyloidosis, possibly unifying all these pathologies under the term 'proteinopathies', where an aberrantly fold protein becomes the 'seeding template' for other normally constituted proteins to misfold and aggregate in intra- and extra-neuronal beta-strand structures, conferring toxicity to selected populations of neurons. Interestingly, recent studies have attempted to unravel the mechanism of spreading the abnormally fold proteins from one neuron to the other. One of the main hypothesis scientific literature is testing now is that extracellular vesicles, which are small plasma membrane-derived spheres containing RNAs, enzymes, signal transduction factors, etc., and acting as transporters of these substances [27], may be the vehicles of toxic proteins, explaining how the disease seeds from very distant sites to others with certain degrees of selectivity.

3. SOD1 protein

3.1. Transcriptional regulation

SOD1 has high and ubiquitous expression; its induction is fine-tuned and modulated by complex intracellular events which probably engage multiple positive and negative regulatory elements acting altogether [28]. Different transcriptional factors were involved in SOD1

constitutive and inducible expression. Among these, the C/EBPs (CCAAT/enhancer-binding proteins) have been demonstrated to be necessary for SOD1 constitutive expression. C/EBP consensus element partially overlaps the Sp1/Egr1 sequence, suggesting that these transcription factors may act in the control of SOD1 gene expression regulation [29]. Furthermore, also the transcription factor CCAAT/enhancer-binding protein delta is involved in the regulation of human SOD1 transcription [30]: In particular, it enhances SOD1 mRNA expression in cisplatin-treated human urothelial carcinoma cell line (NTUB1) via a direct promoter transactivation. The over-expression of Sp1 (Specificity Protein 1) enhances SOD1 basal promoter activity [31].

It has also been demonstrated that cytokines and stress signals (radiation, injury, and oxidative or mechanical stress) can induce the expression of early growth response-1 (Egr1), a nuclear phosphoprotein regulator of transcription. Minc and co-workers [32] demonstrated that SOD1 mRNA level rapidly increased after phorbol-12-myristate-13-acetate (PMA) treatment in HeLa cells, and the region between nucleotides −59 and −48 presents noncanonical consensus recognition sequences for Sp1 and Egr1, and it is bound by Egr1 in response to PMA exposure.

It has been demonstrated that activating protein 1 (AP1) represses SOD1 transcription by sequestrating essential coactivators such as Sp1 [31]. Furthermore, neuronal nitric oxide synthase (nNOS) over-expression causes SOD1 down-regulation of mRNA, protein, and activity levels [33], and that this seems to be caused by both the decreased binding of Sp1 to SOD1 promoter, caused by nNOS interaction with Sp1, and a concomitant increased binding activity of AP1 to the same site.

An increased expression of SOD1 mRNA and protein was reported after the exposure of human HepG2 and HeLa cells to the 2,3,7,8-tetrachlorodibenzo-pdioxin (TCDD), an environmental contaminant and interacting with aryl hydrocarbon receptor (AHR) [34]. They also identified the presence of a xenobiotic responsive element (XRE) in the 5′ -flanking region of human SOD1 gene (between −255 and −238 from the transcription start site), which is responsible for the induction by TCDD. A thyroid hormone (TR) inhibitory element was identified between −157 and +17 of the human SOD1 promoter, and T3 exposure reverses the induction of SOD1 transcription caused by the ROS-producing paraquat and PMA agents [35]. Also, SOD1 promoter is significantly up-regulated by unliganded TRs.

NF-κB was one of the first transcription factors shown redox regulated [36, 37]. Moreover, cell treatment with H_2O_2 triggers the PI3K/Akt cascade, which participates in NF-κB activation and in consequent SOD1 transcriptional induction. Thus, a p65-NF-κB-binding site in the human SOD1 promoter (GGTAAGTCCC) was identified, and Akt-activated NF-κB presents an increased binding to this sequence, mediating the up-regulation of SOD1 expression [38].

Dell'Orco et al. [39] investigated if SOD1 over-expression could be related to transcriptional regulation; in particular, they consider the relationship between the nuclear factor E2-related factor 2 (Nrf2) transcription factor and SOD1 promoter [39]. The dissociation from Keap1 is a prerequisite for Nrf2 translocation to the nucleus. The activated Nrf2 re-localizes into the nucleus where it binds to ARE=adenine/uracil-rich element sequences and activates the expression of different cytoprotective target genes. Moreover, an ARE-binding element,

located between −356 and −330 from the transcription start site and targeted by Nrf2, has been identified in SOD1 promoter [40]. By means of a probe harbouring the SOD1 ARE element, we reported the presence of a dynamic protein-DNA complex, which suggested that after H_2O_2 treatment, the cis-acting ARE sequence in SOD1 promoter plays an important role by recruiting multiple TFs and/or cofactors, probably co-operating to modulate SOD1 mRNA induction under oxidative stress. ChIP assay followed by qRT-PCR with primers designed to flank the ARE element in SOD1 promoter showed that Nrf2 occupancy at the ARE elements in SOD1 promoter did not change after H_2O_2 treatment. We also reported a minimal increased association at the ARE element in NQO1 gene, a well-known Nrf2 gene target, thus indicating that Nrf2 cannot gain access to SOD1 5′-flanking region in a native chromatin context. SOD1 mRNA up-regulation in response to H_2O_2 oxidative treatment might indeed be ascribed to the involvement of other transcriptional factors. These data suggested that SOD1 promoter is not a canonical downstream target of the Nrf2 regulatory network and that Nrf2 may act as a 'constitutive' TF, binding to SOD1 ARE sequence even without stimulus, while H_2O_2 treatment could induce changes in the chromatin conformation with consequent changes in the relationship between the different TFs. In regard to SOD1 gene, it has been wrongly considered a housekeeping gene; however, its inducible transcription is finely regulated by *cis*-acting sequences and the corresponding transactive factors [39].

3.2. Post-transcriptional regulation

Until now, post-transcriptional control mechanisms of SOD1 expression are still unexplored; nevertheless, post-transcriptional control represents a specific and punctual regulatory mechanism for gene expression which can easily modify protein levels in response to extracellular stimuli [41, 42]. Variations in gene expression are often the result of synergy between transcriptional and post-transcriptional regulatory mechanisms [28]. Very few data on post-transcriptional regulation of SOD1 have been reported so far. Two species of SOD1 mRNA with different 3′UTR lengths which produce *in vitro* different quantities of SOD1 protein have been identified. Kilk and colleagues [43] reported that the ability of long mRNA to produce more SOD1 enzyme seems to be related to specific sequences located in the 3′UTR; moreover, they identified the presence of AREs in this region (AUUUA, CUUUA, AUUUG, GUUUUA, AUUUU, and AUUUC). In general, AREs represent the docking sites for different RNA-binding proteins (RBPs), which can affect the metabolism of the target transcripts [44]. Among ARE-binding RBPs, ELAV (or Hu) proteins play a critical role [45]; ELAV family comprises the neuron-specific members HuB, HuC, and HuD [46] and the ubiquitously expressed HuR. ELAV proteins act primarily as positive regulators of gene expression; they can increase the stability and/or translation of target mRNAs whose proteins have fundamental cellular functions.

Given the lack of data on this issue in ALS pathogenesis, Cereda's group [26] focused its attention on the modulation of SOD1 mRNA levels by post-transcriptional mechanisms, in particular via ELAV proteins. Considering that oxidative stress plays a critical role in sporadic ALS, and that it is a trigger of ELAV activation, by treating human neuroblastoma SH-SY5Y cells with 1 mM H_2O_2, a significant up-regulation of SOD1 mRNA levels was reported [47].

Milani and collaborators [47] also analysed the 3'UTR region of SOD1 mRNA and, by means a bioinformatics analysis, they identified within SOD1 3'UTR primary sequence the presence of AREs that are putative ELAV-binding sites. These data were consistent with the results reported by Lopez de Silanes and colleagues [48]; REMSA and immunoprecipitation coupled with real-time qPCR experiments demonstrated that the recombinant HuR protein bound specifically and directly to the ARE-bearing probe of SOD1. In parallel, ELAV accumulates in the cytoplasm; this intracellular relocation is in line with the role of ELAV/HuR as a nuclear-cytoplasmic shuttling protein. Indeed, ELAV's influence on target mRNAs depends on its localization in the cytoplasm, where these proteins stabilize target mRNAs and regulate their translation [49]. Taken together, these data seem to indicate that oxidative stress promotes SOD1 induction probably mediated by ELAV-dependent mechanisms. Moreover, the kinase-triggered phosphorylation of ELAV/HuR is crucial for the modulation of its function and allows it to associate extracellular signals to specific post-transcriptional events elicited by this RBP [50, 51]. Finally, Milani and co-authors [47] also reported an enhanced perinuclear and cytoplasmic expression of ELAV in the cerebral motor cortex of SALS patients compared to healthy subjects, suggesting that the up-regulated RBP proteins may be more available and massively enrolled in the positive regulation of target mRNAs, such as SOD1. Remarkably, positivity for cytoplasmic ELAV was paralleled by increased levels of both SOD1 mRNA and protein [47].

SOD1 post-transcriptional regulation may also be mediated by microRNAs (miRNAs). miRNAs are small non-protein-coding RNAs, which act as key post-transcriptional regulators of gene expression by base pairing to the 3'UTR of the target mRNAs, causing translational silencing and mRNA decay [52, 53], as it was reported for miR-377 in human and mouse mesangial cells which diminished SOD1 protein levels [54].

In consideration of all these findings, we can conclude that the increase in SOD1 gene expression is the consequence of an intricate process related both to the activation of a transcriptional mechanism involving Pol II, and also other mechanisms such as chromatin changes and epigenetic variations, mRNA, and protein stabilization.

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