

Changes in Als2 Expression Leads to Different Outcomes on the Expression of Two NF- κ B Targeted Genes in N2a and C2C12 Cell Lines

Als 2 Ekspresyonunun NF- κ B Hedefli İki Gen Üzerindeki Olası Etkisinin N2a ve C2C12 Hücre Hatlarında Araştırılması

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ABSTRACT

Objective: Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder affecting spinal and cortical motor neurons. Motor neuron degeneration leads to progressive muscular atrophy. Als2 (alsin) is one of the causative genes of ALS and UXT gene is an interacting partner of Als2. Since UXT is a known cofactor in NF- κ B enhancesome, we aimed here to compare the effects of Als2 silencing on NF- κ B activity by using N2a and C2C12 cell lines.

Methods: Mouse neuroblastoma cells (N2a) and myoblast cell lines (C2C12) were used. Als2 was silenced by RNAi and UXT, A20 and IL8 expressions were measured by qRT-PCR. Data were statistically evaluated by Student t-test.

Results: A 73.4% decrease was observed in Als2 expression in N2a cells, whereas 73.7% decrease in the C2C12 cells was found. UXT expression in Als2-silenced N2a cells was found to be decreased by 40%, while a 9-fold increase in C2C12 cells was detected. IL8 expression demonstrated a 84% decrease in N2a cells. On the other hand, IL8 expression increased 50-fold in C2C12 cells. A20 expression increased by 65% in N2a cell line, and increased 10 fold in C2C12 cell line.

Conclusion: We have concluded that silencing Als2 gene over UXT on exerts different effects on NF- κ B pathway. We arrived at this conclusion because changes in the expressions of the IL8 and A20 genes differ in both cell lines. In addition, the link between Als2 and UXT is shown again in both cell lines and it is understood that the silencing Als2 may give rise to different results as for neuronal cells and muscle cells.

Keywords: ALS, Als2, NF- κ B, N2a, C2C12

ÖZ

Amaç: Amiyotrofik Lateral Skleroz (ALS) spinal ve kortikal motor nöronları etkileyen nörodegeneratif bir hastalıktır. Motor nöron dejenerasyonu ise kaslarda ilerleyici atrofiye neden olur. ALS'ye neden olan genlerden biri Als2 (alsin) genidir ve UXT geni de Als2 ile etkileşimde olan genlerden biridir. UXT proteininin NF- κ B kompleksinde kofaktör olarak rol aldığı da bilinmektedir. Bu çalışmadaki amacımız, Als2'nin susturulmasının NF- κ B aktivitesindeki olası etkilerini N2a ve C2C12 hücre hatlarında karşılaştırmaktır.

Yöntem: Bu çalışmada, fare nöroblastoma hücreleri (N2a) ile fare miyoblast hücreleri (C2C12) kullanıldı. Söz konusu hücrelerde Als2 geni RNAi ile susturuldu ve UXT, IL8 ve A20 genlerinin ekspresyonları qRT-PCR ile ölçüldü. Veriler Student t-test kullanılarak istatistiksel olarak değerlendirildi.

Bulgular: N2a hücrelerinde Als2 ekspresyonundaki azalış %73,4 olurken C2C12 hücrelerinde %73,7'lik bir düşüş gözlemlendi. Als2 geni susturulmuş N2a hücrelerinde UXT seviyesi %40 azalırken, C2C12 hücrelerinde 9 katlık bir UXT artışı saptandı. IL8 ekspresyonu N2a hücrelerinde %84'lük bir düşüş gösterdi, ancak C2C12 hücrelerinde IL8 ekspresyonu 50 katlık bir yükseliş kaydetti. Öte yandan A20 ekspresyonu N2a hücre hattında %65 artarken, bu artış C2C12 hücre hattında 10 misli olarak gerçekleşti.

Sonuç: Als2 geninin susturulmasının UXT üzerinden NF- κ B yolağına olan etkilerinin farklı olduğu sonucuna ulaşılmıştır. Bu sonuca IL8 ve A20 genlerinin ekspresyonlarındaki değişimlerin her iki hücre hattında da farklılık göstermesi üzerinden varılmıştır. Ayrıca Als2 ve UXT arasındaki bağlantı her iki hücre hattında da tekrar gösterilmiş ve Als2'nin susturulmasının nöronal hücreler ile kas hücreleri açısından farklı sonuçlar doğurabileceği de anlaşılmıştır.

Anahtar kelimeler: ALS, Als2, NF- κ B, N2a, C2C12

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INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative condition leading to motor neuron loss. Degeneration of motor neurons causes progressive wasting of skeletal muscles and muscular atrophy, and death ensues within 2-5 years of diagnosis. Ten per cent of the ALS cases have hereditary transmission and the rest is sporadic¹. One of the proposed pathological mechanisms underlying ALS pathogenesis is the “dying back” phenomenon, which emphasizes that the cell death starts from the neuromuscular junction, spreads along the axon and eventually arrives at the neuronal cell body^{2,3}. This hypothesis is mainly based on the assumption that neuropathological changes are caused by the alterations of the neuromuscular junction, which in turn, are induced by the pathological changes of the muscle. Recent studies about the “dying back” phenomenon have indicated that during the early stages of the ALS pathogenesis in *Sod1*(G93A) transgenic mice nerve terminals and neuromuscular junctions are found to be degraded while the soma of the motor neurons located in the spinal cord gray matter remain intact. In addition, impaired mitophagy has been described in the neuromuscular junctions of the *Sod1*(G93A) transgenic mice implying the possible role of mitochondrial involvement in these very special subcellular regions^{4,5}.

In addition to this hypothesis, excitotoxicity, oxidative stress, abnormal energy metabolism, dysfunction of axonal transport and inflammation have also been proposed as candidate mechanisms for the degeneration of motor neurons⁶.

ALS2 (alsin), one of the causative genes of ALS, is located on chromosome 2q33.1. Mutations of *ALS2* leading to a premature termination of translation or to amino acid substitution, cause an autosomal recessive type of ALS with juvenile onset through the loss of function of the alsin protein. The alsin protein contains several functional domains including three putative Guanine Nucleoti-

de Exchange Factor (GEF) domains, the regulator of chromosome condensation 1 (RCC1) domain, the Dbl-homology/Pleckstrin-homology (DH/PH) domain and the Vacuolar Protein Sorting 9 (VPS9) domain^{7,8}.

Recent scientific literature exhibits that the alsin protein may play a role in endosomal trafficking and that it could also function in intracellular signalling with its DH/PH domain⁹. The possible neuroprotective function of alsin has also been proposed in degenerating motor neurons in which mutant *SOD1* is expressed¹⁰⁻¹².

Our group has already found that the Ubiquitously Expressed Transcript gene (*Uxt*) is an interacting partner of *Als2*^{3,14} and it is known that the UXT protein is a chaperon belonging to α -class prefoldin-like family proteins¹⁵. This interaction has also been documented in primary spinal motor neurons and the interaction has an effect on the two NF- κ B-dependent genes (*IL8* and *A20*) through *Uxt*¹⁶.

As a complementary study we here generated *Als2* knock-down stable cell lines of neuronal N2a and myoblast C2C12 cells and aimed to investigate the changes in the expression levels of *Uxt* and NF- κ B activated genes, *IL8* and *A20*.

MATERIALS and METHODS

Plasmid constructs

The full open reading frame of the mouse *Als2* transcript was amplified by using the following primers:

Als2(FL)F: 5'GGACTCAAAGAAGAAAAGCTCAA
CAGA 3'

Als2(FL) R: 5' TTACAACCAAAATAGCACAAA-
AGTCCA 3'

The PCR product of the full length *Als2* was cloned first into the pTZR57R/T vector by using “InstaClone PCR Cloning Kit” (Fermentas, Canada) and then transferred to the mammalian expressi-

on vector pCRUZ-GFPTM (Santa Cruz, USA), using EcoRI and SmaI sites.

In order to generate short hairpin RNA (shRNA) specific for the DH/PH region of the *Als2* transcript, we used the pSilencerTM2.1-U6 (Ambion, ABI, USA) vector. The following oligos were annealed and cloned into the vector according to the manufacturer's instructions.

Als2(KD)F: 5'GATCCATGACGGATTCCTTGAGGAT TCAAGAGATCCTCAAGGAATCCGTCATTTTTTTGG AAA 3'

Als2(KD)R: 5'AGCTTTTCCAAAAAATGACGGAT TCCTTGAGGATCTCTTGAATCCTCAAGGAATCCGT CATG 3'

Cell Culture Assays

Neuronal N2a cells were purchased from ATCC (USA) (Catalog no: CCL-131). They were grown and propagated in Minimal Essential Medium (MEM) (GIBCO, USA) containing 10% (v/v) Foetal Bovine Serum (GIBCO, USA), 200 mM Glutamine (GIBCO, USA), 1% (v/v) Non-Essential Amino Acids (GIBCO, USA) and 100U:100 μ g Penicillin/Streptomycin cocktail (GIBCO, USA) in a cell culture incubator. The C2C12 cell line was also purchased from ATCC (USA) (Catalog no: CRL-1772), and it was propagated in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, USA), containing 10% (v/v) Foetal Bovine Serum (GIBCO, USA), 200 mM Glutamine (GIBCO, USA), 1% (v/v) Non-Essential Amino Acids (GIBCO, USA) and 100U:100 μ g Penicillin/Streptomycin cocktail (GIBCO, USA). Because the *Als2* knock-down expression plasmids contain hygromycin- resistance gene for selection, the optimum hygromycin (Sigma, USA) concentration was determined as 600 μ g/ml by using the antibiotic titration method for both cell lines. Then neuronal N2a and myoblast C2C12 cells were grown until 60% confluency was reached, and they were stably transfected with 2 μ g expression vector pSilencerTM2.1-U6 (Ambion, ABI, USA), containing shRNA, specific for the *Als2* transcript or control vector having scrambled

shRNA sequence provided by the manufacturer in OPTIMEM (GIBCO, USA) and FuGENE HD Transfection Reagent (Roche, Germany), according to the manufacturer's instructions. After 72 hours of transfection, OPTIMEM was replaced with complete growth medium and 600 μ g/ml hygromycin was added to the medium every three days for antibiotic selection of stably transfected cells. At the end of day 10 of hygromycin selection, the *Als2* gene expression was measured.

In order to overexpress *Als2* transiently in both cell types, pCRUZ-GFPTM (Santa Cruz, USA), the mammalian expression vector containing *Als2* open reading frame was used and the recommended transfection protocol of FuGENE HD Transfection Reagent (Roche, Germany) was followed.

Quantitative RT-PCR (qRT-PCR)

In order to determine the expression levels of *Als2*, *Uxt*, *IL8* and *A20*, total RNA was isolated from the cells by using the High Pure RNA Isolation Kit (Roche, Germany). QRT-PCR was performed using the LightCycler RNA Master SYBR Green I Kit and LightCycler 2.0 (Roche, Germany). The following primers were used:

*Als2*F: 5' TCCAGTTCTTGCTATGAGTCTCT 3'

*Als2*R: 5' GGAATCCGTCATTTTCCAGG 3'

*Uxt*F: 5' TTGGGCTGTAACCTCTTCGGTTG 3'

*Uxt*R: 5' AGGAGAGAAGCTTTTCGGTCAA 3'

*IL8*F: 5' ATGCCCTCTATTCTGCCAGAT 3'

*IL8*R: 5' GTGCTCCGGTTGTATAAGATGAC 3'

*A20*F: 5' TGGGTGCCCTTTACTTTGAAT 3'

*A20*R: 5' GCTCTGCTGTAGTCCTTTTAAA 3'

β -*Actin*F: 5' GGCTGTATCCCCTCCATCG 3'

β -*Actin*R: 5' CCAGTTGGTAACAATGCCATGT 3'

QRT-PCR experiments were repeated at least three times independently and the β -Actin transcript level was used to normalize the data according to $\Delta\Delta$ Cq method. Student-t test in the Statistical Package of Social Sciences (SPSS) Version 22 software was used to measure the statistical significance of the fold changes seen in the data. When

the p value was found to be less than 0.05, the fold changes were considered statistically significant. To test the normality of the data, Anderson-Darling (AD) test was implemented. In order to implement the normality test AD-Test Calculator developed by the University of Missouri, was used as a free excel-based software¹⁷. When the p value was found to be greater than 0.05, then it was considered that the normality of the data was attained.

RESULTS

Alterations of Uxt Expression when Als2 expression was changed

After neuronal N2a and C2C12 myoblast cells were stably transfected with pSilencerTM2.1-U6 (Ambion, ABI, USA), containing shRNA specific for the DH/PH region of the *Als2* transcript, total RNA isolation was performed; and followed by qRT-PCR in order to check the *Als2* transcript levels. In neuronal N2a cells, a significant (73.4%) decrease in *Als2* transcript was observed with respect to the control cells ($p < 0.0001$), whereas a statistically significant reduction of 73.7% was found in the C2C12 myoblast cells ($p < 0.0001$) (Fig. 1a and b). In both cell lines *Uxt* expression was measured using the same method. In *Als2* knock-down background of neuronal N2a cells, *Uxt* expression was found to be significantly dec-

reased by 40% ($p < 0.0001$), while in *Als2* knock-down C2C12 myoblasts *Uxt* expression increased 9-fold ($p < 0.0001$) (Fig. 2a and b).

We also performed experiments of *Als2* overexpression in both cell lines to check if the *Uxt* expression changed. It was found that when *Als2* was overexpressed transiently in both neuronal N2a and C2C12 myoblasts, *UXT* expression was statistically significantly upregulated ($p < 0.0001$) (Fig. 2c and d).

3.2. Changes of the Expression of NF- κ B activated genes when Als2 was downregulated

Knowing that the *Uxt* has a role in the activity of NF- κ B activated genes such as IL8 and A20, these two genes have been chosen to elucidate the possible effect of *Als2* silencing on the NF- κ B pathway through the alterations of *Uxt* expression. In *Als2* knocked-down neuronal N2a cells, IL8 expression was found to be decreased significantly by 84% ($p < 0.0001$), on the other hand, in *Als2* knocked-down C2C12 myoblasts, it significantly increased by approximately 50-fold ($p < 0.0001$) (Fig. 3a and b). A20 expression was found to be upregulated by only 65% ($p < 0.0001$) in *Als2* knocked-down neuronal N2a cells, whereas it was drastically increased (10-fold) in *Als2* knocked-down C2C12 myoblasts ($p < 0.0001$) (Fig. 3c and d).

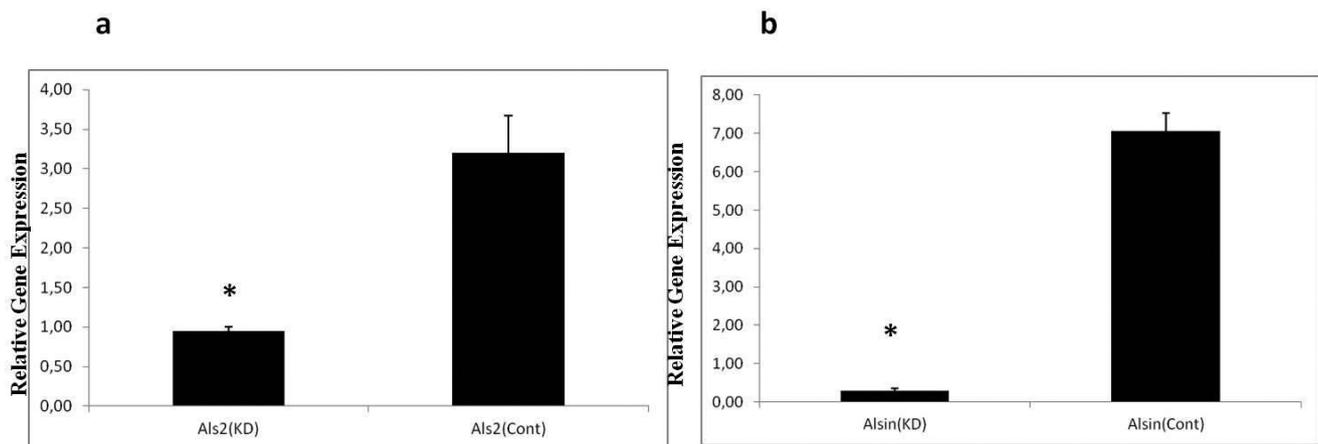


Figure 1. Effect of Als2 silencing on Uxt expression in neuronal N2a (a) and C2C12 myoblast cell lines (b). (*) symbol indicates that $p < 0.0001$ and bars indicate standard deviations (SD).

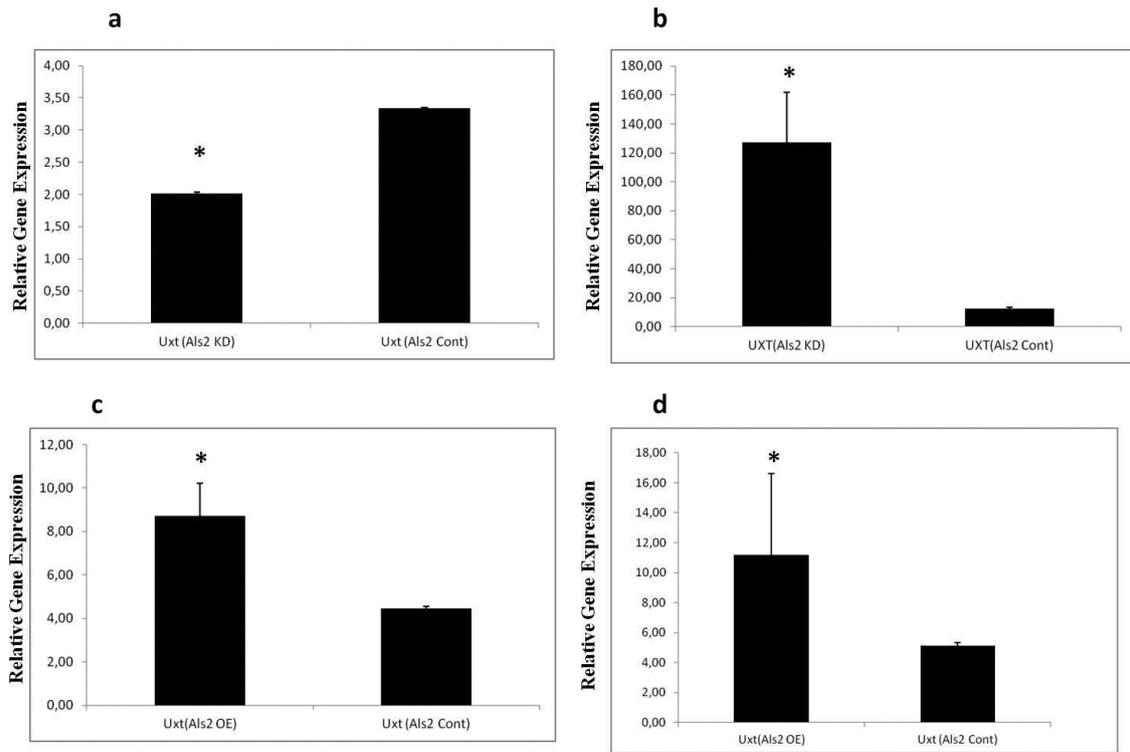


Figure 2. Effect of Als2 silencing on Uxt expression in N2a cells (a) and in C2C12 myoblasts (b). Effect of Als2 overexpression on Uxt expression in N2a cells (c) and in C2C12 myoblasts (d). (*) symbol indicates that $p < 0.0001$ and bars indicate standard deviations (SD).

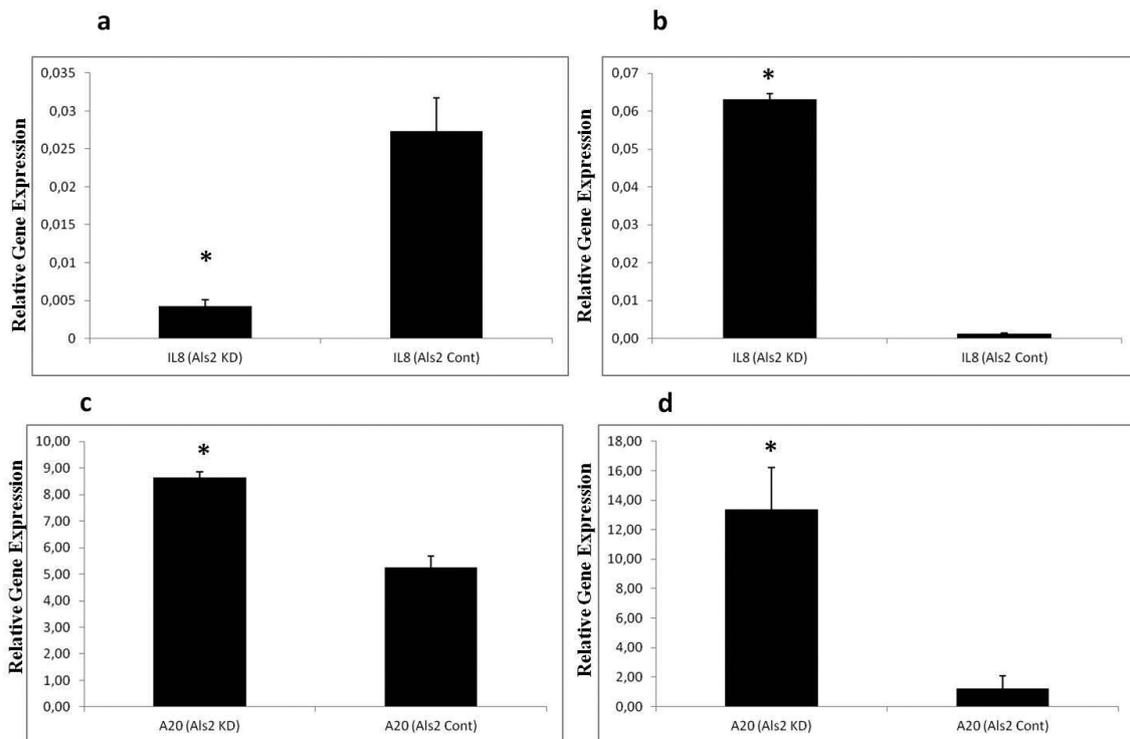


Figure 3. Effect of Als2 silencing on IL8 expression in N2a cells (a) and in C2C12 myoblasts (b). Effect of Als2 silencing A20 expression in N2a cells (c) and in C2C12 myoblasts (d). (*) symbol indicates that $p < 0.0001$ and bars indicate standard deviations (SD).

DISCUSSION

Although a set of genes responsible for the development of ALS, when mutated, has been described so far, the underlying molecular mechanisms remain to be elucidated. The critical fact concerning ALS pathology is that the degenerative process occurring in motor neurons starts at the neuromuscular junction; this phenomenon (dying-back) leads some researchers to consider that not only motor neurons but also the muscles which these motor neurons innervate should also be taken into account¹⁸.

ALS2, one of the causative genes of ALS, encodes a protein, which has several functional domains, still the exact function of the ALS2 protein is not well understood. Knowing that the Als2 protein interacts with UXT, and UXT is a cofactor in NF- κ B enhancesome complex, in this study we aimed to search for the possible effects of *Als2* gene silencing on NF- κ B activity. The gene expression analyses above reveal that neuronal N2a cells and C2C12 myoblasts respond differently to Als2 knock-down in terms of UXT gene expression. These responses were followed by investigating the expression levels of *IL8*, and *A20*.

Although *A20* expression was found to be upregulated in both cell lines used in this study; the amount of this increase was quite different. In N2a cells *A20* was upregulated at a rate of 65%, on the other hand, in C2C12 cell line it was upregulated 10-fold. Knowing that *A20* has a regulatory activity on NF- κ B activity and apoptotic responses, this difference may be linked to the notion that the pro-apoptotic cellular events would be boosted in myoblasts when *Als2* is silenced, while the *A20*-related pro-apoptotic activation is moderate in N2a cells¹⁹⁻²¹. The behaviour of the *IL8* expression is totally different. In N2a cell line it is downregulated in the *Als2* knock-down background, however, in C2C12 cells *IL8* expression increases very dramatically.

These findings show that not only the *Als2* protein interacts with Uxt as it was previously shown¹³, but also the *Als2* gene expression affects the Uxt gene expression both in N2a cells and C2C12 myoblasts.

From the perspective of the “dying back phenomenon” our findings indicate that the inflammatory alterations would start at the muscle tissue and the degenerative changes may spread from the muscle to the motor neuron via neuromuscular junction^{22,23}. Also in both our *Als2* silenced cell lines pro-apoptotic gene expression was represented by increases in *A20* gene expression but in myoblasts it increased dramatically, which would lead us to consider that the initiation of apoptotic events may also start from the muscle.

Altogether these results imply that inflammatory and pro-apoptotic alterations could both be present in motor neurons and skeletal muscles, but in order to reach more definitive conclusions about the starting site of degenerative processes seen in ALS, co-culturing studies of neuronal and skeletal muscle cell lines will be implemented, or co-culturing of primary spinal motor neurons with primary skeletal muscle cells will be performed²⁴⁻²⁷.

CONCLUSION

In these cell lines, responses to *Als2* knock-down in terms of UXT gene expression were different. These responses were followed by investigating the expression levels of *IL8*, and *A20*. Our findings show that not only the *Als2* protein interacts with UXT, but the *Als2* gene expression affects the UXT gene expression both in N2a cells and C2C12 myoblasts.

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