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RESEARCH ARTICLE

Spinal cord extracts of amyotrophic lateral sclerosis spread TDP-43 pathology in cerebral organoids

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Abstract

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder caused by progressive loss of motor neurons and there is currently no effective therapy. Cytoplasmic mislocalization and aggregation of TAR DNA-binding protein 43 kDa (TDP-43) within the CNS is a pathological hallmark in sporadic ALS and prion-like propagation of pathogenic TDP-43 is thought to be implicated in disease progression. However, cell-to-cell transmission of pathogenic TDP-43 in the human CNS has not been confirmed experimentally. Here we used induced pluripotent stem cells (iPSCs)-derived cerebral organoids as recipient CNS tissue model that are anatomically relevant human brain. We injected postmortem spinal cord protein extracts individually from three non-ALS or five sporadic ALS patients containing pathogenic TDP-43 into the cerebral organoids to validate the templated propagation and spreading of TDP-43 pathology in human CNS tissue. We first demonstrated that the administration of spinal cord extracts from an ALS patient induced the formation of TDP-43 pathology that progressively spread in a time-dependent manner in cerebral organoids, suggesting that pathogenic TDP-43 from ALS functioned as seeds and propagated cell-to-cell to form de novo TDP-43 pathology. We also reported that the administration of ALS patient-derived protein extracts caused astrocyte proliferation to form astrogliosis in cerebral organoids, reproducing the pathological feature seen in ALS. Moreover, we showed pathogenic TDP-43 induced cellular apoptosis and that TDP-43 pathology correlated with genomic damage due to DNA double-strand breaks. Thus, our results provide evidence that patient-derived pathogenic TDP-43 can mimic the prion-like propagation of TDP-43 pathology in human CNS tissue. Our findings indicate that our assays with human cerebral organoids that replicate ALS pathophysiology have a promising strategy for creating readouts that could be used in future drug discovery efforts against ALS.

full-length TDP-43 to induce cytoplasmic TDP-43 inclusion formation [34]. Consistent with these reports, GR repeat proteins were seen in OrgALS cerebral organoids injected with protein extracts from C9orf72-ALS, but not from non-C9orf72-ALS. In addition, pTDP-43 aggregates colocalized with GR repeat proteins in OrgALS cerebral organoids injected with protein extracts from C9orf72-ALS, suggesting that GR repeat protein spread and recruited the formation of TDP-43 pathology in cerebral organoids that do not have the *C9orf72* gene expansion (S4B Fig). This result reproduces the C9orf72 pathology where GR repeat protein colocalizes with pathological TDP-43 inclusions in the motor cortex of C9orf72-ALS patient [35].

Taken together, our results indicated that ALS patient-derived protein extract containing pTDP-43 were pathogenic seeds that spread TDP-43 pathology in cerebral organoids, especially cerebral organoids from a patient who had ALS, recapitulating the pathogenic TDP-43 propagation phenomenon in human CNS tissue.

ALS patient-derived protein extracts cause astrogliosis in cerebral organoids

Astrocytes are key components of the CNS that are involved in multiple neural homeostatic functions. They influence synaptic function and formation, regulate the concentration of neurotransmitters at the synapse, supply metabolites to neurons, provide neurotrophic factors, and aid in the repair of damaged neural tissue [36–38]. ALS pathology displays astrogliosis correlated to astrocyte proliferation and hypertrophy in the CNS, including spinal cord, cerebral cortex, and subcortical white matter [39]. Therefore, we investigated whether the formation of TDP-43 pathology is accompanied by astrogliosis in cerebral organoids. Immunofluorescence analysis showed that, while OrgCtrl cerebral organoids did not show GFAP-positive astrocyte proliferation, OrgALS cerebral organoids exhibited an increasing amount of astrocytes after they were injected with ALS patient-derived protein extracts (Fig 4A and 4B). The statistical analysis of the immunofluorescence signal demonstrated that GFAP-positive astrocytes were significantly proliferated in OrgALS cerebral organoids that were injected with ALS patient-derived protein extracts (Fig 4C). To confirm the immunofluorescence results, we further investigated the expression of GFAP in cerebral organoids using Western blot analysis. While the amount of GFAP expression was not different between OrgCtrl cerebral organoids injected with control or ALS patient-derived protein extract, a significant difference in GFAP expression was seen in OrgALS cerebral organoids (Fig 4D and 4E). Densitometry analysis of Western blot results confirmed this finding (Fig 4F and 4G). These data demonstrate that ALS patient-derived protein extracts containing pTDP-43 induced astrogliosis as well as TDP-43 pathology in cerebral organoids of an ALS patient.

ALS patient-derived protein extracts induce cellular apoptosis and DNA double-strand breaks

Recent studies show that TDP-43 pathology correlates with neural apoptosis due to DNA double-strand break repair defects [40–42]. Thus, we next examined the cytotoxic effects of TDP-43 pathology and DNA damage in cerebral organoids injected with ALS patient-derived protein extracts.

We first performed TUNEL stain assay to evaluate cellular apoptosis in cerebral organoids. Few TUNEL-positive cells were seen in OrgCtrl cerebral organoids after the administration of protein extracts from either control or ALS patient (Fig 5A). However, OrgALS cerebral organoids had several TUNEL-positive cells after injection with ALS patient-derived protein extracts (Fig 5B). Statistical analysis confirmed the increase in TUNEL-positive cells in OrgALS cerebral organoids injected with ALS patient-derived protein extract (Fig 5C). We

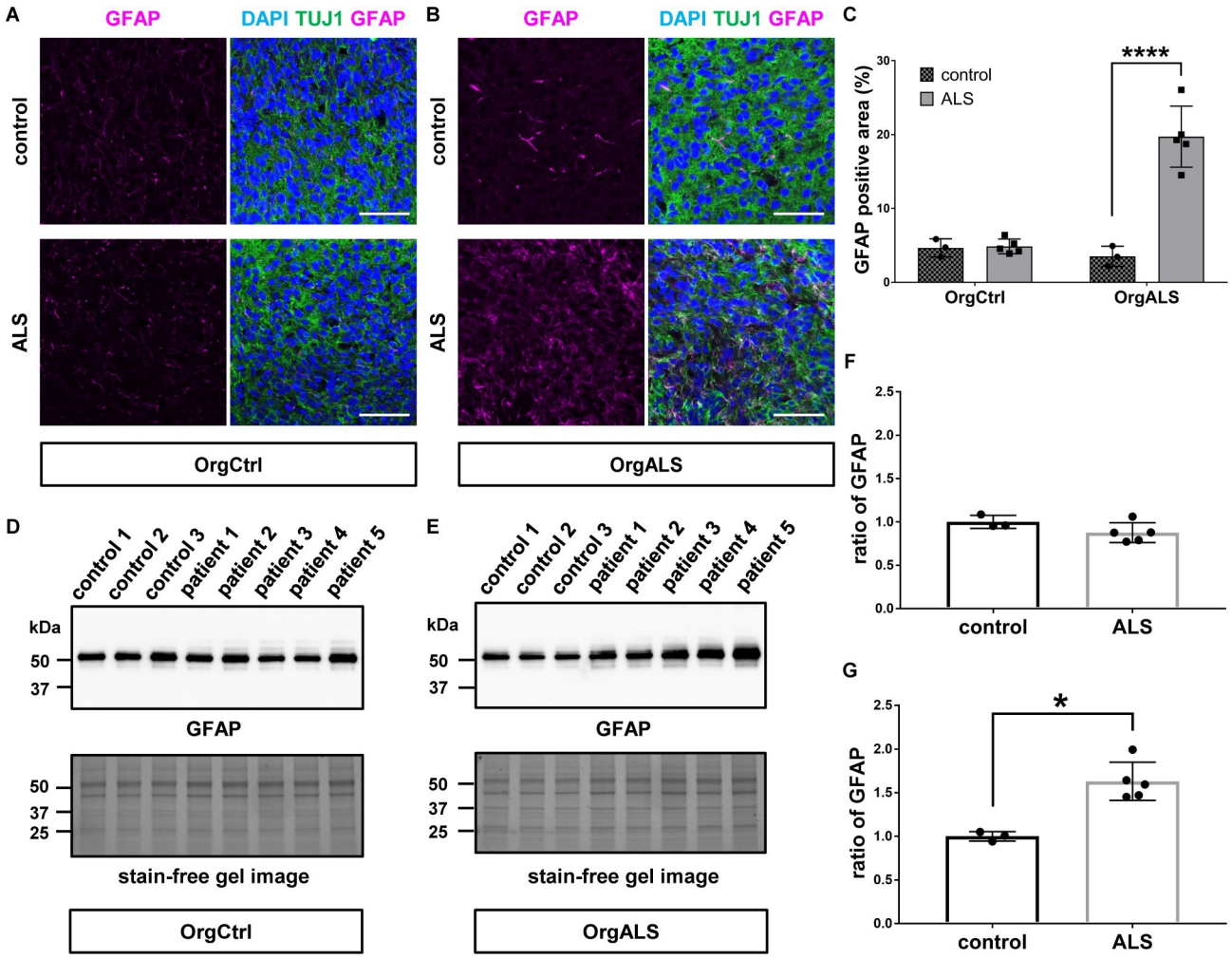

Fig 4. ALS patient-derived protein extract injection induces astrogliosis in cerebral organoids. (**A** and **B**) Immunofluorescence images of GFAP and TUJ1 staining OrgCtrl (**A**) or OrgALS cerebral organoids (**B**) at 8 weeks p.i. of protein extract from control (control 1) or ALS (patient 3). DAPI was used for counterstaining of nuclei. Scale bars = 50 μm. (**C**) Quantification analysis of immunofluorescence images of GFAP staining OrgCtrl and OrgALS cerebral organoids at 8 weeks p.i. of protein extract from individual controls (control 1–3) or ALS cases (patient 1–5) represented in (**A**) and (**B**). Percentages of GFAP-positive area were measured with ImageJ software. Bar plots show mean ± SD with individual points representing a different proteins extract. Differences were evaluated by two-way ANOVA with Tukey's multiple comparisons test. $^{****}p<0.001$. (**D** and **E**) Western blot analysis of GFAP expression in OrgCtrl (**D**) or OrgALS cerebral organoids (**E**) at 8 weeks p.i. of protein extracts from individual controls (control 1–3) or ALS cases (patient 1–5). A stain-free gel image was used for protein loading controls. (**F** and **G**) Densitometric quantification of Western blot analysis in (**D**) and (**E**), respectively, using ImageJ software. Each data point was obtained by normalization to bands in stain-free gel blot images. Bar plots show mean ± SD with individual points representing a different protein extract from controls (control 1–3) and ALS (patient 1–5). Differences were evaluated by unpaired *t*-test. $^{*}p<0.05$.

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next investigated the expression of cleaved caspase-3 protein, a marker of cellular apoptosis. In immunofluorescence analysis, OrgCtrl cerebral organoids had little cleaved caspase-3, whether they were injected with control or ALS patient-derived protein extracts (Fig 5D). On the other hand, the expression of cleaved caspase-3 was increased in OrgALS cerebral organoids after injection with ALS patient-derived protein extracts (Fig 5E). Statistical analysis confirmed that cleaved caspase-3 was significantly higher in OrgALS cerebral organoids that received ALS patient-derived protein extracts (Fig 5F). Moreover, the cleaved caspase-3-positive cells contained pTDP-43 aggregates in OrgALS cerebral organoids after injection with ALS patient-derived protein extracts (Fig 5G). Using Western blot analysis, we further investigated the

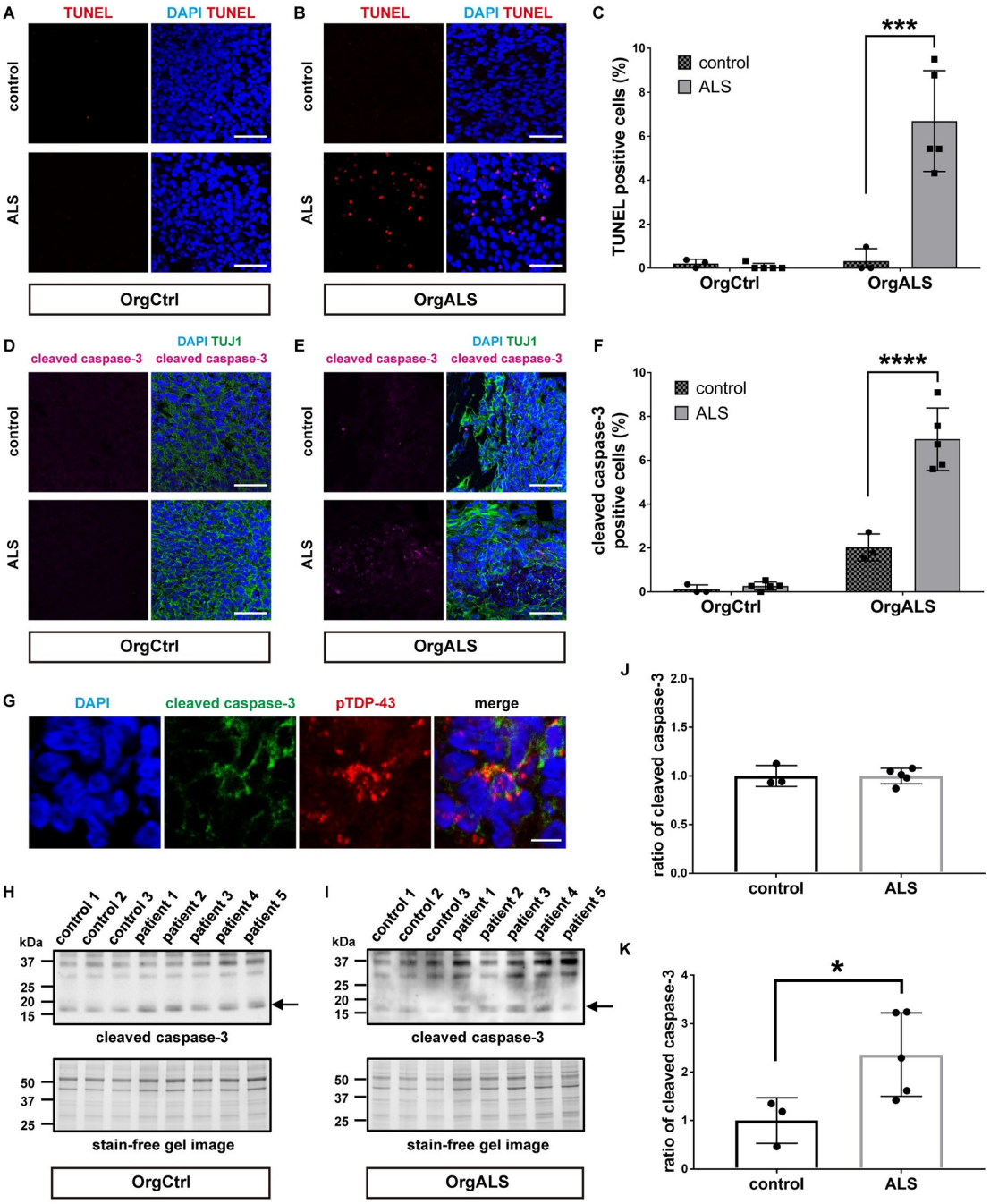

Fig 5. ALS patient-derived protein extracts cause cellular apoptosis in cerebral organoids. (**A** and **B**) TUNEL stain images of OrgCtrl (**A**) or OrgALS cerebral organoids (**B**) at 8 weeks p.i. of protein extract from control (control 1) or ALS (patient 4). DAPI was used for counterstaining of nuclei. Scale bars = 50 μm. (**C**) Quantification analysis of TUNEL stain images of OrgCtrl and OrgALS cerebral organoids at 8 weeks p.i. of protein extract from individual controls (control 1–3) or ALS cases (patient 1–5) representatively shown in (**A**) and (**B**). Bar plots show mean ± SD with individual points representing a different protein extract. Percentages of TUNEL-positive cells were normalized to number of DAPI-positive cell nuclei. Differences were evaluated by two-way ANOVA with Tukey's multiple comparisons test. $^{***}p<0.005$. (**D** and **E**) Immunofluorescence images of cleaved caspase-3 and TUJ1 staining OrgCtrl (**D**) or OrgALS cerebral organoids (**E**) at 8 weeks p.i. of protein extract from control (control 1) or ALS (patient 5). Scale bars = 50 μm. (**F**) Quantification analysis of cleaved caspase-3 staining immunofluorescence images of OrgCtrl and OrgALS cerebral organoids at 8 weeks p.i. of protein extract from individual controls (control 1–3) or ALS cases (patient 1–5) represented in (**D**) and (**E**). Bar plots show mean ± SD with individual points representing a different protein extract. Percentages of cleaved caspase-3-positive cells were normalized to number of DAPI-positive cell nuclei. Differences were evaluated by two-way ANOVA with Tukey's multiple comparisons test. $^{****}p<0.001$. (**G**) Immunofluorescence images of cleaved caspase-3 and pTDP-

43 (pS409/410) staining OrgALS cerebral organoids at 8 weeks p.i. of protein extract from ALS (patient 5). Scale bars = 10 μm. (**H** and **I**) Western blot analysis of cleaved caspase-3 expression in OrgCtrl (**H**) or OrgALS cerebral organoids (**I**) at 8 weeks p.i. of protein extracts from individual controls (control 1–3) or ALS (patient 1–5). A stain-free gel image was used for protein loading controls. Arrows indicate cleaved caspase-3 protein bands detected in approximately 17 kDa molecular weight. (**J** and **K**) Densitometric quantification of Western blot analysis in (**J**) and (**K**), respectively, using ImageJ software. Bar plots show mean ± SD with individual points representing a different protein extract. Each data point was obtained by normalization to bands in stain-free gel blot images. Differences were evaluated by unpaired *t*-test. $^{*}p<0.05$.

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amount of cleaved caspase-3 expressions in our models. As was found by immunofluorescence, there was no difference in cleaved caspase-3 expression levels in OrgCtrl cerebral organoids injected either with control or ALS patient-derived protein extracts, while in OrgALS cerebral organoids there was more cleaved caspase-3 expression with ALS patient-derived protein extracts (Fig 5H and 5I). The statistical evaluation using densitometry in Western blot analysis also revealed that cleaved caspase-3 expression was the same in OrgCtrl cerebral organoids injected with control or ALS patient-derived protein extract, but it was significantly increased in OrgALS cerebral organoids injected with ALS patient-derived protein extracts (Fig 5J and 5K). These results indicate that ALS patient-derived protein extracts containing pathogenic TDP-43 trigger neural apoptosis in ALS-derived cerebral organoids.

Recent studies uncovered the relationship between TDP-43 pathology and DNA damage which causes neural apoptosis. They reported that pathological TDP-43 induces DNA double-strand breaks (DSBs) repair defects in ALS [41,42]. DSBs evoke phosphorylation of histone variant protein H2AX on serine 139 [43]. The phosphorylated H2AX protein, gamma-H2AX (γH2AX), forms nuclear foci which recruit DNA repair proteins. Hence the formation of γH2AX nuclear foci is widely used as a specific marker to evaluate DSBs [44]. Based on these previous reports, we investigated by immunofluorescence whether the cellular apoptosis caused by pathogenic TDP-43 in cerebral organoids was associated with DNA damage by detecting γH2AX. OrgCtrl cerebral organoids showed only a few γH2AX nuclear foci whether they were injected with extracts from control or ALS patient-derived protein extracts (Fig 6A). In contrast, OrgALS cerebral organoids had more γH2AX nuclear foci after they were injected with ALS patient-derived protein extract than from control protein extract (Fig 6B). Moreover, some γH2AX colocalized with cytoplasmic pTDP-43 aggregates in OrgALS cerebral organoids, suggesting that pathogenic pTDP-43 recruited DSBs repair proteins with γH2AX in the cytosol (Fig 6B). Statistical analysis confirmed these findings (Fig 6C). We next performed Western blot analysis to measure the expression of γH2AX. Expression levels were not different in OrgCtrl cerebral organoids between controls and ALS patient-derived protein extracts, and expression was slightly higher in OrgALS cerebral organoids injected with ALS patient-derived protein extracts than in the control injected organoids (Fig 6D and 6E). The densitometry of Western blot results showed that, while there was no difference for the ratio of γH2AX expression between controls and ALS in OrgCtrl cerebral organoids, injection of ALS patient-derived protein extracts in OrgALS cerebral organoids significantly enhanced γH2AX expression compared to controls (Fig 6F and 6G).

Overall, our data demonstrates that pathogenic TDP-43 from sporadic ALS patient-derived protein extracts has the potential to seed and propagate in ALS-derived cerebral organoids used as human CNS tissue model, inducing astrogliosis and cellular apoptosis concomitant with DSBs.

Discussion

We demonstrate that ALS patient-derived protein extracts containing pathogenic TDP-43 have the potential to spread cell-to-cell and to form phosphorylated TDP-43 cytoplasmic

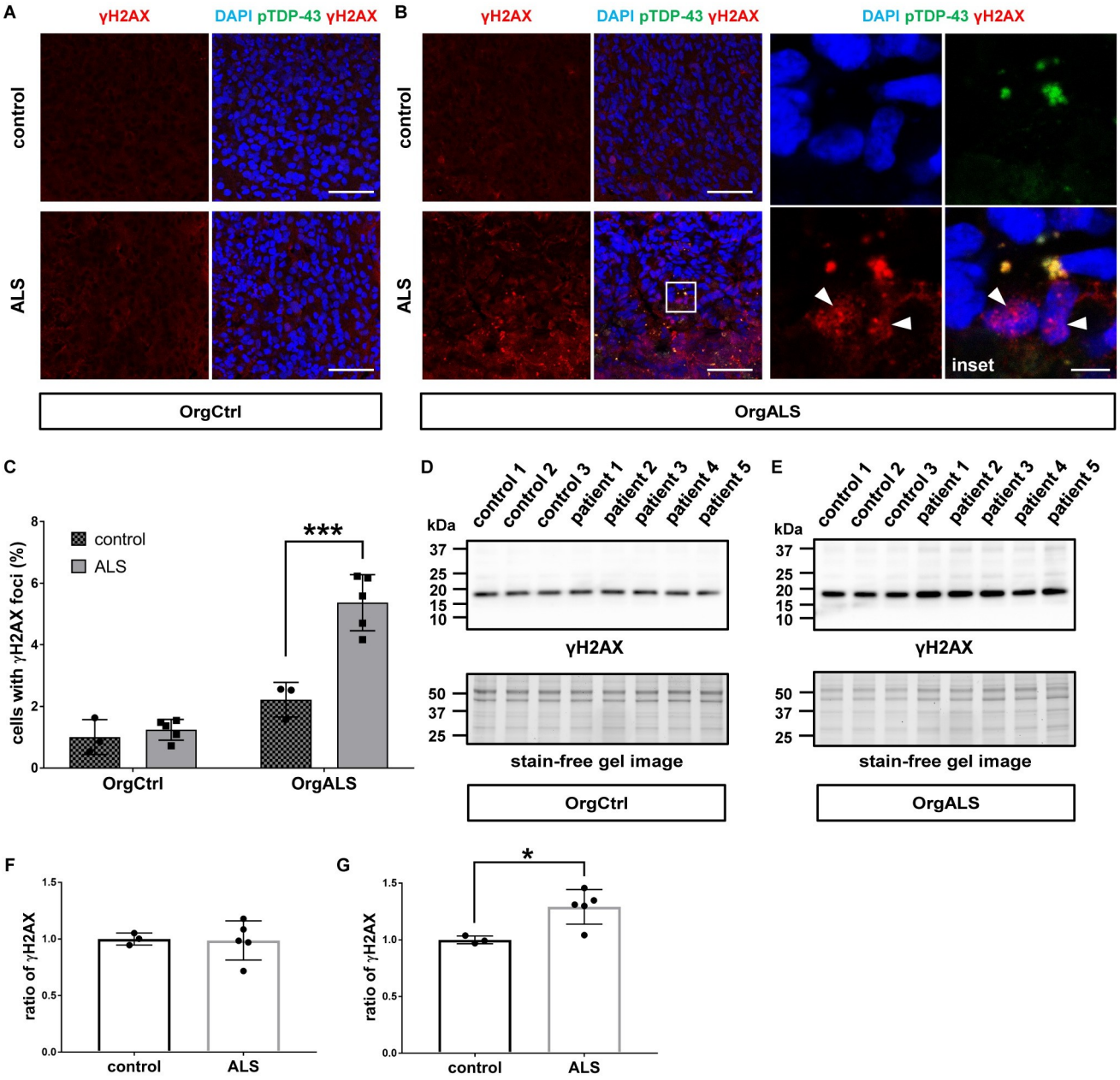

Fig 6. TDP-43 pathology promotes DNA damage in cerebral organoids. (**A** and **B**) Immunofluorescence images of γH2AX and pTDP-43 (pS409/410) staining OrgCtrl (**A**) or OrgALS cerebral organoids (**B**) at 8 weeks p.i. of protein extract from control (control 1) or ALS (patient 2). A higher magnification of white line box is shown in the right inset. DAPI was used for counterstaining of nuclei. Arrow heads indicate γH2AX nuclear foci. Scale bars = 50 μm (10 μm for inset). (**C**) Quantification analysis of γH2AX staining immunofluorescence images of OrgCtrl and OrgALS cerebral organoids at 8 weeks p.i. of protein extract from individual controls (control 1–3) or ALS cases (patient 1–5) represented in (**A**) and (**B**). Bar plots show mean ± SD with individual points representing a different protein extract. Percentages of cells harboring γH2AX nuclear foci were normalized to number of DAPI-positive cell nuclei. Differences were evaluated by two-way ANOVA with Tukey's multiple comparisons test. $^{***}p<0.005$. (**D** and **E**) Western blot analysis of γH2AX expression in OrgCtrl (**D**) or OrgALS cerebral organoids (**E**) at 8 weeks p.i. of protein extracts from individual controls (control 1–3) or ALS (patient 1–5). A stain-free gel image was used for protein loading controls. (**F** and **G**) Densitometric quantification of Western blot analysis in (**D**) and (**E**), respectively, using ImageJ software. Bar plots show mean ± SD with individual points representing a different protein extract from controls (control 1–3) and ALS (patient 1–5). Each data point was obtained by normalization to bands in stain-free gel blot images. Differences were evaluated by unpaired *t*-test. $^{*}p<0.05$.

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aggregates replicating ALS pathology in a time-dependent manner in recipient human cerebral organoids. We also showed that the TDP-43 pathology causes astrogliosis, cellular apoptosis and genomic damage. Our findings support the recent reports describing that pathogenic TDP-43 show prion-like propagation in murine brain and spinal cord of TDP-43 transgenic animal administrated with ALS patient-derived tissue [15,16] and that TDP-43 pathology correlates with DSBs *in vitro* and *in vivo* [41,42]. These results were relatively consistently seen with ALS patient-derived protein extracts from five different individuals. Ours is the first study to elucidate the seeding activity of patient-derived pathogenic TDP-43 and the formation of TDP-43 pathology in a human CNS tissue model.

We use mature iPSCs-derived cerebral organoids as a model of the human brain. These organoids have a complex three-dimensional structure with a wide variety of neuronal and glial cell types, that resembles human brain tissue.

Interestingly, the cerebral organoids derived from sporadic ALS-FTLD patient (OrgALS cerebral organoids), but not cerebral organoids from a control individual, showed increased cytoplasmic pTDP-43 aggregates and the TDP-43 pathology as early as two weeks after the administration of ALS patient-derived protein extracts. Cerebral organoids differentiated from an unaffected healthy control (OrgCtrl cerebral organoids) formed small amounts of cytoplasmic pTDP-43 accumulation only eight weeks after the injection. Our findings raise the possibility that cells from someone who developed ALS carry some factors making them more susceptible to TDP-43 induced pathology. All known genetic factors have been ruled out as a possible cause for this susceptibility. Perhaps some unknown genetic factors, or some residual epigenetic marks, are responsible for this susceptibility to prion-like spread of pathogenic TDP-43. Based on the experiments for TDP-43 propagation in transgenic mice, Porta *et al.* [16] describe that both concentration and subcellular localization of TDP-43 protein are important for TDP-43 aggregation and that disturbances in nuclear-cytoplasmic TDP-43 protein homeostasis may play a role in TDP-43 nucleation and aggregation. Although our cerebral organoids from iPSCs derived from an ALS-FTLD patient do not exhibit apparent cytoplasmic TDP-43 expression before the administration of ALS patient-derived protein extract, they may have a defect in nuclear-cytoplasmic TDP-43 translocations that facilitates TDP-43 aggregation once exposed to pathogenic TDP-43 from ALS.

Albeit we did not discriminate directly between exogenous and endogenous TDP-43 in recipient cerebral organoids, our results showing the reduction of nuclear TDP-43 and the time-dependent increase of pTDP-43 aggregates in the cerebral organoids administrated with ALS patient-derived protein extracts suggest that exogenous pathogenic TDP-43 acts as seeds and propagates cell-to-cell to form de novo TDP-43 pathology by altering normal endogenous TDP-43 to pathological conformations. The addition of a protein marker such as a Flag-tag to TDP-43 protein extracts may provide the evidence more clearly that exogenous pathological TDP-43 can template endogenous TDP-43. Although the specific mechanisms for prion-like propagation of pathogenic TDP-43 remains enigmatic, accumulating evidence indicates that exosomes play a role in transferring several pathological proteins associated with neurodegenerative disorders such as tau, α-synuclein, and SOD1 [45–47]. As for TDP-43, a previous report demonstrates that secreted exosomes from ALS brain causes cytoplasmic TDP-43 distribution in neural cultured cells, suggesting that exosomes may contribute to propagation of TDP-43 pathology [48]. Although it remains unclear whether exosome-mediated transfer is a major pathway for TDP-43 propagation in ALS, our study using cerebral organoids and patient-derived protein extract has the potential to be a useful model for evaluating molecular factors that cause TDP-43 transmission.

Astrogliosis characterized by hypertrophy and proliferation of astrocytes as well as upregulation of GFAP expression is one of the key features observed in ALS pathology [39]. Our

model successfully reproduces the astrogliosis seen in ALS. Of note, astrocytes are involved in both familial and sporadic ALS pathogenesis by releasing various toxic factors that provoke neuroinflammation and neurotoxicity [49–51]. The increase in activated astrocytes in our ALS cerebral organoid model may contribute to cellular death and spread of misfolded TDP-43 via secreted neurotoxic molecules.

We show here that the administration of ALS patient-derived protein extracts also induces cellular apoptosis and DSBs in recipient cerebral organoids, suggesting a link between apoptotic cellular death and genomic damage in TDP-43 pathology. Genome damage-mediated neuronal apoptosis in ALS has been reported in several studies. TDP-43 is a critical component of the DSBs repair pathway and physiologically acts as scaffold for the recruitment of DSBs repair proteins at DSB sites. Loss of nuclear TDP-43 as well as ALS-linked TDP-43 mutations are associated with DSB repair defects that trigger neuronal apoptosis in ALS [40–42]. Moreover, genomic instability due to DNA repair defects and DSBs are also seen in C9orf72-ALS [52]. Thus, our findings are consistent with these previous reports, indicating that loss of TDP-43 physiological function as a scaffold for DSBs repair proteins due to nuclear depletion of normal TDP-43 is associated with genomic damage and cytotoxicity in cerebral organoids.

There are some limitations to this study. We used only two cell lines of human iPSCs to create recipient cerebral organoids. Additional iPSCs lines, especially some derived from ALS cases, are required to provide a better understanding of pathogenic TDP-43 propagation in cerebral organoids and possible host factors. Moreover, given that ALS is a late-onset disease, our cerebral organoids at around 10–20 weeks growth *in vitro* do not contain as many mature neurons as an adult human. The ideal cerebral organoids would need to have achieved the same level of maturity as adult CNS tissue, an objective that has yet to be achieved, would be preferable to verify pathogenic TDP-43 propagation. We used cerebral organoids as a CNS tissue model resembling the human brain, while another critical site for TDP-43 pathology in ALS is the spinal cord. In addition, microglia derived from mesoderm, which is an important factor involved in ALS pathophysiology, is deficient in cerebral organoids composed only of cells differentiated from ectoderm. However, robust protocols for creating well-structured spinal cord organoids harboring microglia have not yet been established. It is necessary to wait for such protocols that create organoids that reproduce the anatomical structures and physiological functions of human spinal cord tissue to verify our results.

In conclusion, our findings provide evidence that pathogenic TDP-43 in ALS spinal cord tissue has the potential to spread cell-to-cell to induce TDP-43 pathology as well as astrogliosis and to cause cell cytotoxicity due to DNA damage in human CNS tissue. Although additional investigation is required to elucidate the key factors that induce pathogenic TDP-43 transmission, our model could be used to test treatment strategies aimed at blocking of pathogenic TDP-43 propagation.

Supporting information

S1 Table. Human iPSCs lines information used in this study. Human iPSCs used in this study were generated from peripheral blood mononuclear cells (PBMCs) of a healthy control (AJC001 line; OrgCtrl) or sporadic ALS-FTLD patient (TD17 line; OrgALS). N.A. indicates "not assessed".
(PDF)

S2 Table. Human tissue information used in this study. Postmortem frozen spinal cords tissue specimens were collected from the Montreal Neurological Institute-Hospital (MNI), the Douglas-Bell Canada Brain Bank (DBCBB) and the London Neurodegenerative Diseases Brain

Bank (LNDBB). N.A. indicates "not assessed".
(PDF)

S3 Table. Antibody information used in this study. IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blot; DB, dot blot; N.A. indicates "not assessed".
(PDF)

S1 Fig. Sporadic ALS-FTLD patient of OrgALS iPSCs line displays TDP-43 pathology in anterior horn spinal cords. (**A and B**) Immunohistochemistry of anterior horn spinal cords stained by TDP-43 antibody. The blue arrow shows punctate intraneuronal inclusion (**A**) and the orange arrow shows skein-like intraneuronal inclusion (**B**). Scale bar = 50 μm.
(TIF)

S2 Fig. Cerebral organoids exhibit neural cortices-like tissue mimic to human brain. (**A**) Representative bright-field (*left*) and immunofluorescence images (*right*) of cerebral organoids differentiated from OrgCtrl or OrgALS iPSCs line at 60 DIV. Scale bars = 1 mm. (**B**) Double-label immunofluorescence images of CTIP2 and PAX6 staining OrgCtrl or OrgALS cerebral organoids at 30 DIV and day 60 DIV. Scale bars = 100 μm. (**C**) Double-label immunofluorescence images of CTIP2 and TUJ1 (*upper panels*) or SOX2 (*lower panels*) staining OrgCtrl or OrgALS cerebral organoids at 60 DIV. Sections were counterstained with DAPI to label the nuclei. Scale bars = 50 μm.
(TIF)

S3 Fig. pTDP-43 aggregates distribute in astrocytes. Immunofluorescence images of OrgALS cerebral organoid double-labelled with pTDP-43 and GFAP at 8 weeks post injection of protein extracts from ALS (patient 5). The lower panels are higher magnifications of the white-line boxes in the upper panels. Scale bars = 50 μm (*upper panel*) and 10 μm (*lower panel*).
(TIF)

S4 Fig. pTDP-43 aggregates colocalize with GR repeat. (**A**) Dot blot analysis of sarkosyl-insoluble protein extracts from non-ALS control (control 1) and C9orf72-ALS spinal cords (patient 3), immunoblotted with GR repeat antibody. (**B**) Immunofluorescence images of OrgALS cerebral organoids double-labelled with pTDP-43 and GR repeat proteins at 8 weeks post injection of protein extracts from C9orf72-ALS (patient 3) (*upper panels*) or non-C9orf72-ALS (patient 4) (*lower panels*). Scale bars = 10 μm.
(TIF)

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