University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
August 2019

Characterizing TDP-43 Proteinopathy in Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that primarily affects upper and lower motor neurons. The pathology of ALS associates closely with cellular level physiological abnormalities in TAR DNA binding protein (TDP-43), a ubiquitously expressed, highly conserved nuclear protein. TDP-43 proteinopathy involves excessive mislocalization and misfolding within the cytoplasm where certain components of the protein aggregate into hyper-phosphorylated tau-negative ubiquitinated inclusions. These intracellular alterations impair neurite growth during neuronal differentiation while damaging cell viability and ultimately causing apoptotic necrosis in affected tissues. Studying the effects of ALS induced TDP-43 aggregation *in vitro* requires the establishment of a neuronal cell culture that recapitulates the physiology of human motor neurons. NSC-34 cells can be differentiated to model selected aspects of motor neuron development. In this study, an NSC-34 cell culture system is established for the future comparison of cells transiently transfected for regulated expression of both wild-type and C-terminal mutated (Q311K) TDP-43 proteins. The results indicate that NSC-34 cells seeded onto a 96-well plate in serum-free media optimally differentiate motor neuron-like morphology, as measured by intra-well neurite length, when plated at 10k cells/well.

1. Introduction

1.1 Amyotrophic Lateral Sclerosis

As an insidious and ultimately fatal neurodegenerative disease, amyotrophic lateral sclerosis (ALS) principally induces dysfunction and degeneration within populations of affected upper and lower motor neurons in the cerebral cortex, brain stem, and spinal cord.[1,2] Chronic impairment of motor signaling causes muscle atrophy and progressive paralysis in affected patients.[3] Fatality frequently follows respiratory failure.[4] ALS also shares pathobiological similarities with frontotemporal dementia (FTD) and both diseases are co-diagnosed in nearly a sixth of ALS cases.[5] The symptoms of FTD overlap with ALS symptomatology as approximately half of the ALS patient population develops cognitive and behavioral impairment in addition to chronic motor deficits.[6]

Clinical heterogeneity complicates both epidemiological studies and medical treatment of ALS. Pathological abnormalities at the cellular level may precede clinical manifestations of disease, potentially for months or years, while the clinical presentation of the disease progression may differ significantly depending on a variety of genetic and environmental factors.[4] The incidence of ALS ranges from one to three per 100,000, with an average age of onset around 60 years. Familial cases resulting from heritable genetic mutations may present themselves much earlier.[7] Life expectancy following diagnosis ranges from one to five years, after which less than a tenth of patients survive.[8]

In the absence of curative therapies, current treatment for ALS attempts to decelerate disease progression while alleviating symptoms; however, available medications are minimally effective for these purposes. The pharmaceutical antiglutamate agent riluzole prolongs median survival by only two to three months but enables diagnosed patients to retain motor and cognitive function for a statistically significant length of time.[9,10] Adverse reactions to the medication

include asthenia, spasticity, and mild elevations in aminotransferase levels.[11] Non-invasive respiratory support likewise improves survival and quality of life[12]. Mechanical interventions that bypass the upper airways using a treacheostomy or endotracheal tube extend survival for years; however, these methods fail to slow disease progression.[13]

1.2 TAR DNA binding Protein

As with other neurodegenerative diseases, complex interactions between genetic factors and molecular pathways are thought to generate multifactorial pathophysiological mechanisms for ALS.[14] Nearly a tenth of cases appear to be familial, with an autosomal dominant pattern of inheritance, while the remainder arise sporadically.[1,15] Familial ALS (FALS) most frequently results from defects in chromosome 9 open reading frame 72 (C9ORF72) and from mutations in the enzyme copper-zinc superoxide dismutase 1 (SOD1); these targets have been studied extensively and their role in disease incidence is well established.[16,17]

More recent literature suggests that some FALS phenotypes associate closely with physiological abnormalities in TAR DNA binding protein (TDP-43), a ubiquitously expressed, highly conserved nuclear protein capable of binding both DNA and RNA to regulate gene transcription and RNA splicing; the protein also contributes to other RNA processing events such as somatodendritic transport.[18,19] Critically, under normal conditions TDP-43 regulates the stability of its own mRNA through a negative feedback loop.[20] Despite the diverse etiology of the disease, 97% of ALS patients show evidence of aberrant TDP-43 deposition in affected populations of upper and lower motor neurons.[21] Pathological TDP-43 deposition is also a common feature frontotemporal dementia, lending credence to the possibility that both diseases share similar pathophysiological mechanisms.[22]

Although most TDP-43 localizes within the nucleus, the protein may also engage in nucleocytoplasmic shuttling.[23] Nearly a third of cellular TDP-43 normally resides within the cytoplasm with nuclear efflux regulated in part by the influence of environmental stressors.[24,25] Under conditions of oxidative insult, TDP-43 assembles into stress granules (ribonucleoprotein complexes) that function to sequester nonessential mRNA transcripts; TDP-43 thereby contributes to neuronal plasticity by regulating somatodendritic protein synthesis.[19,25]

1.3 TDP-43 Proteinopathy

TDP-43 proteinopathy involves excessive mislocalization and misfolding within the cytoplasm where certain components of the protein aggregate into hyper-phosphorylated tau-negative ubiquitinated inclusions with associated depletion of TDP-43 from the nucleus.[18] Although covalent binding of ubiquitin normally marks proteins for degradation, failure to clear ubiquitinated proteins seems to potentiate macroaggregation and disrupt cellular homeostasis.[26] Mutated TDP-43 appears especially prone to abnormal cleavage, phosphorylation, ubiquitination and subsequent aggregation.[18]

The presence of ubiquitinated inclusions correlates closely with neuronal atrophy and cell death.[24] Studies on the brains of deceased patients show that the regional spread of ubiquitinated TDP-43 inclusions accurately stages ALS progression, lending support to the hypothesis that pathological TDP-43 deposition propagates between affected cells along axonal pathways.[27] This mechanism is consistent with TDP-43's involvement in somatodendritic transport.[19]

Pathological aggregates containing TDP-43 may sequester the protein into dysfunctional stress granules that burden affected neurons through a toxic gain of function; alternatively, mislocalized or misfolded TDP-43 may fail to interact with appropriate binding partners and deprive the cell of essential activities due to a loss of function.[21] These effects may be particularly insidious with regards to TDP-43 since pathologically aggregated proteins fail to provide autoregulatory negative feedback, thereby facilitating excessive protein production and deposition.[20]

1.4 Animal and Cellular Models

Transgenic mice (*Mus musculus*) overexpressing wild-type TDP-43 develop spastic paralysis and neuronal inclusions in a toxic gain of function model consistent with ALS and FTD pathology.[28] Similarly, transgenic flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*) demonstrate progressive neurodegeneration and functional deficits that recapitulate the clinical features of TDP-43 proteinopathy.[29,30] Loss of function models using TDP-43 knockout mice result in embryonic lethality and fail to demonstrate adult-onset neurodegeneration.[31,32,33] In tandem, these studies suggest that neurodegenerative TDP-43 proteinopathy originates from a toxic gain of function rather than from any functional deficiencies.

Cellular models of TDP-43 proteinopathy likewise demonstrate a toxic gain of function *in vitro*. Mammalian NSC-34 cells engineered to both full length and low molecular weight C-terminal TDP-43 fragments between 35 to 25 kDa recapitulate neurodegenerative phenotypes.[34] Both wild-type and mutated TDP-43 overexpression impairs intracellular mitochondrial function in stably transfected NSC-34 cells by decreasing their mitochondrial transmembrane potential, reducing expression of mitochondrial complex I activity, and increasing expression of mitochondrial uncoupling protein 2.[35] Full length TDP-43 and its C-terminal fragments accumulate within transfected NSC-34 mitochondria before inducing dysfunction and mitophagy, as indicated by changes in protein-markers LC3-II and p62 that are reliably associated with autophagosomes.[36]

1.5 Proposed Studies

Studying the effects of ALS induced TDP-43 aggregation *in vitro* requires the establishment of a neuronal cell culture that recapitulates the physiology of human motor neurons. NSC-34 is an immortalized hybrid cell line formed by fusing murine aminopterin-sensitive neuroblastoma N18G2 with motor neuron enriched embryonic spinal cord cells.[37] Under serum deprivation these cells can be induced to model selected aspects of motor neuron development including neurite outgrowth and action potential generation in addition to cholinergic and glutaminergic activity.[38,39] Under optimized conditions the cellular differentiation process may be monitored using confocal microscopy.[40] This experiment addresses the optimal seeding density of NSC-34 cells for study using confocal microscopy by measuring neurite extension as an indicator of neuronal differentiation.

Previous literature suggests that TDP-43 aggregation impairs neurite extension during neuronal differentiation, damages cell viability, and ultimately causes apoptotic necrosis in affected tissues.[34] Mutations associated with familial ALS frequently occur on the intrinsically disordered C-terminal region of TDP-43.[15] Domains on the C-terminus bind heterogeneous ribonuclear proteins and fragments of this region have a high aggregation propensity.[41,34] This experiment attempts to demonstrate structural pathology in NSC-34 cells transiently transfected for regulated expression of wild-type and C-terminal mutated TDP-43 proteins (Q311K) by analyzing the effect on neurite extension and cell viability as measured through confocal microscopy and a cell death assay. Overexpression of both TDP-43 variants should recapitulate the cellular pathology of ALS by reducing intra-well neurite length and increasing the rate of cell death.

2. Methodology

2.1 Proliferation

NSC-34 cells were purchased from CedarLane Cellutions Biosystems Inc. Proliferative NSC-34 cells were thawed and plated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were split at a 1:10 dilution every 3-4 days.

2.2 Differentiation

To differentiate the proliferative cells into motor neuron-like cells, the media was exchanged every two or three days and replaced with differentiative media (1:1 DMEM/F-12 [Ham], 1% FCS, 1% modified Eagle's medium nonessential amino acids [NEAA], and 1% P/S). Differentiated cell populations were viable for approximately three weeks over the course of the experiment.

2.3 Confirmation

In order to confirm that the NSC-34 cells were differentiating as expected and to optimize their seeding conditions, differentiated cells were quantified using a hemocytometer, and seeded into a 96-well plate at 2.5K, 5K, 7,5K, and 10K cells per well. Cells were monitored in real time using the Incucyte® S3 Live Cell Analysis System. The IncuCyte analysis software was trained to recognize and quantify neurites within our laboratory's specific cell culture conditions. After four days, the differentiated NSC-34 wells were analyzed for average neurite length to determine which seeding density produced optimal differentiation.

2.4 Transfection

Coding sequences for wild-type TDP-43 (DNASU HsCD00079870) were mutated (Q311K) through site directed mutagenesis, and subcloned into Pf12 RM Flexi® Vectors (Promega) using Sgfl and Pmel restriction enzymes. eGFP acted as a visual confirmation for successful transfection. The pReg neo Vector (Promega) was utilized to regulate TDP-43 protein expression within transfected cells. Transfection complexes containing the aforementioned plasmids were formed using the ViaFect™ Transfection Reagent in Opti-MEM Reduced Serum Media at a concentration of 1 µg of DNA per 100 µl of media with a 1:3 ratio of DNA:ViaFect and incubated at room temperature for 10 minutes. 10 µl of the newly formed transfection complex solution was pipetted into each well of a 96-well plate seeded with 10K differentiated NSC-34 cells per well. Transfection was confirmed using eGFP fluorescence as a visual marker.

2.5 Regulation of Protein Expression

A chimeric transactivator protein expressed by the pReg neo Vector (Promega) interacts with a regulatory promoter on the Pf12 RM Flexi® Vectors (Promega) to regulate TDP-43 protein expression in response to two aminocoumarin antibiotic compounds. The pReg neo Vector (Promega) contains an SV40 early promoter with six tandem repeats of the λ operator and a minimal CMV promoter. Coumermycin A1 causes dimerization of the transactivator protein and allows λ repressor domains to interact with λ operator sequences on the Pf12 RM Flexi® Vectors (Promega), thereby increasing TDP-43 protein expression. Novobiocin inhibits the actions of coumermycin A1 and was included in experimental lanes to test for potential toxicity.

2.6 Imaging and Cell Death Assay

Images acquired via microscopy were assessed for green fluorescence as a measure of transfection efficiency and for morphological changes including cell count and number of visible neurites. Annexin V-FITC Kit (Invitrogen™) staining would be analyzed using a plate reader to measure colorimetric changes indicative of cell death.

3. Results

3.1 Differentiation

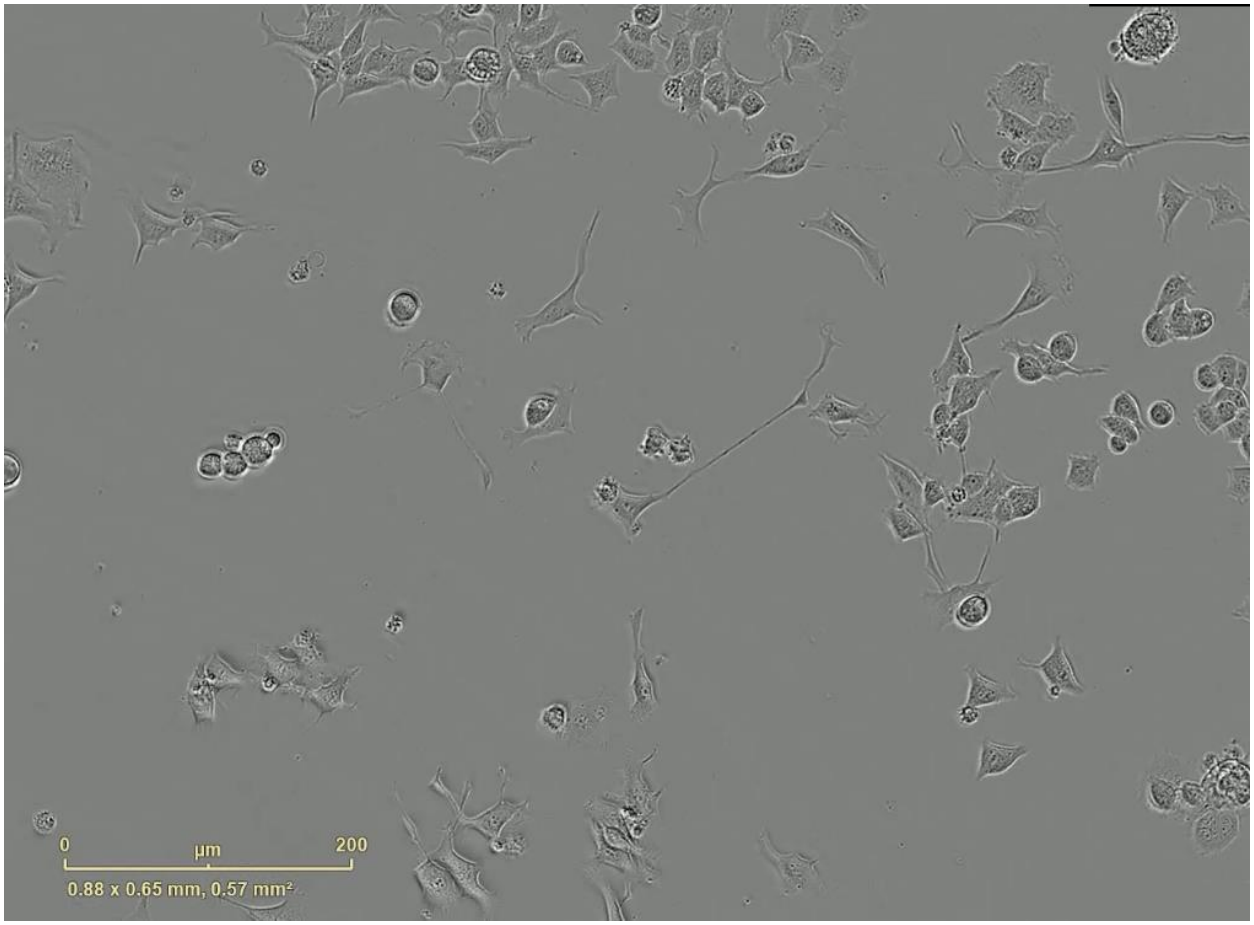

Figure 1. NSC-34 neurite extensions.

Figure 1 An image of differentiating NSC-34 cells with visible neurite extensions was captured using Incucyte® S3 Live Cell Analysis System.

NSC-34 cells exist as two morphologically distinct populations; neuroblastic cells have few cytoskeletal projections and proliferate rapidly whereas mature motor-neuron like cells recapitulate many aspects of motor neuron development including neurofilament expression and neurite outgrowth (Fig. 1).[37] Mature motor neuron-like cells represent a better model for neurodegenerative experiments and their differentiation should be optimized for neurodegenerative experimental models.[40] The tendency of neuroblastic populations to differentiate can be modulated by adjusting the cell culture conditions such as the serum content of culture media and the initial cell seeding density of each well. The results of this experiment indicate that NSC-34 cells seeded onto a 96-well plate in serum-free media optimally differentiate motor neuron-like morphology, as measured by intra-well neurite length, when plated at 10k cells per well (Fig. 2).

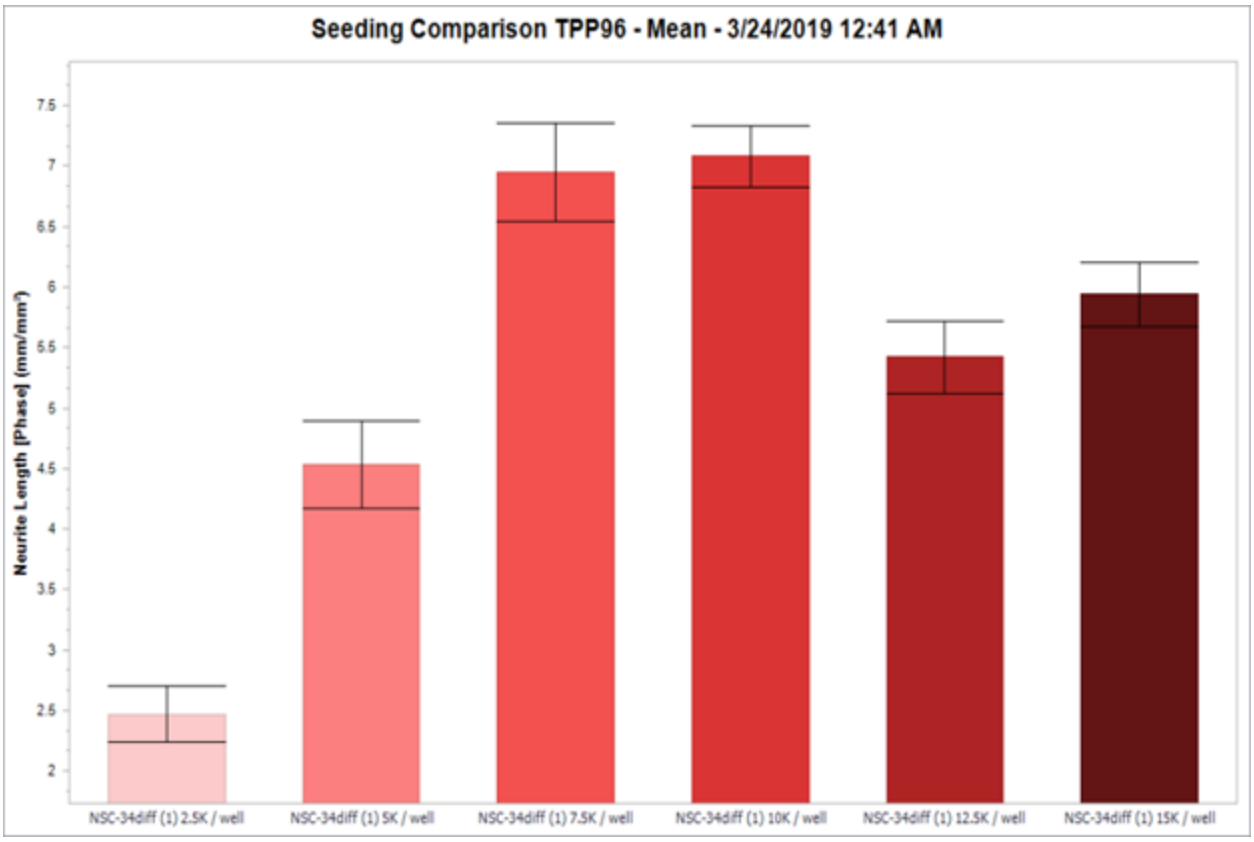

Figure 2. NSC-34 Differentiation

Figure 2 NSC-34 cells demonstrated optimal differentiation, as measured by Incucyte® Live Cell Analysis System according to average intra-well neurite length, when seeded at 7.5K and 10K cells per well in a 96-well plate.

3.2 Transfection

The transfection experiments (Fig. 3) were inconclusive as the plated cells suffered an infection. The first lane without any treatments would have established baseline cell counts and apoptosis levels as a control for transfection. The presence of eGFP in the second lane would have served as a confirmation of transfection and produce a transfected population of cells which could be compared to the control lane to monitor for any potential toxicity from the ViaFect Transfection Reagent; annexin staining would be analyzed with fluorescent microscopy and by using a plate reader to measure colorimetric changes indicative of cell death. The third and eighth lanes was transfected with all three vectors but lacked coumermycin or novobiocin treatment to register the effect of basal TDP-43 WT and Q311K protein expression. The remaining lanes contained all treatments in addition to either coumermycin or novobiocin to ascertain differences in cell morphology or viability as a result of increased TDP-43 protein expression and to test for the antibiotics' potential toxicity.

Figure 3. Transfection plate map.

Figure 3 A schematic representation of the experimental plate map. Lane 1 contained differentiated NSC-34 cells at 10K cells per well without any transfection treatments. Lane 2 was transfected with eGFP alone. Lane 3 was transfected with wild-type TDP-43 in the absence of coumermycin or novobiocin. Lanes 4 and 5 were transfected with wild-type TDP-43 and protein expression was upregulated using coumermycin at 2.5 µg/well, while in lanes 6 and 7 wild-type TDP-43 protein expression was downregulated using novobiocin at 50µg/well. Lane 8 was transfected with mutated TDP-43 (Q311K) without coumermycin or novobiocin. Lanes 9 and 10 were transfected with TDP-43 Q311K and likewise treated with coumermycin, while lanes 11 and 12 were treated with novobiocin. Annexin was present in all lanes at 33 µl / well.

4. Conclusion

This experiment attempted to optimize the seeding density of NSC-34 cells by measuring neurite extension as an indicator of neuronal differentiation. Undifferentiated NSC-34 cells lack the characteristic structural and physiology features of motor neurons which makes them a poor model for studying the potential TDP-43 induced neurotoxicity associated with ALS. Increasing the proportion of differentiated cells in culture can increase the quality and reliability of data obtained by future experiments on this cell line. The results indicate that seeding density substantially impacts the ability of proliferative NSC-34 cells to differentiate; cells plated at sub-optimal seeding densities exhibit marked reductions in neurite outgrowth, which is an important structural biomarker of maturation (Fig 3).

Reductions in neurite outgrowth are a pathological feature of ALS.[42] Given that seeding density also alters neurite outgrowth for plated NSC-34 cells, failing to control for seeding density as a variable could confound the results of future experiments. Undifferentiated NSC-34 cells show higher vulnerability to neurotoxins and may respond differently to various experiment reagents.[39] Further control and optimization of NSC-34 differentiation *in vitro* may improve the accuracy of experimental results. Future research could recapitulate these results by analyzing the expression of neuronal and cholinergic markers of NSC-34 cell maturation, such as MAP2, GAP-43 and ChAT, at various seeding densities.[39]

The transfection experiments may be improved by first performing optimization experiments to ascertain the ideal reagent concentrations, as it is unknown whether some of the reagents exhibit toxicity within the proposed experimental model. These variables include controlling for the total concentration of DNA within each well and by adjusting the ratio of DNA to Viafect™ Transfection Reagent used to form transfection complexes. Coumermycin and novobiocin also present some potential for toxicity.[43,44] Optimizing these procedures may improve cell viability in future experiments.

5. Acknowledgements

The author wishes to express his appreciation to Dr. Angel Kaur and to the UNC Asheville Biology Department for the opportunity to pursue this research. Completing this section of the project was possible thanks to contributions from fellow research students, including Rosie Garris, Tatiana Gonzalez, Hannah Henken, Zach Kerkenbush, Leona Wrenn, Mikayla Zopfi, and others.

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