様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　６ 年　 ６ 月 ２７ 日現在

機関番号：３８００５
研究種目：基盤研究(C)（一般）
研究期間：2020～2023
課題番号：２０Ｋ０７４５８

研究課題名（和文）Axonal local translation and its implications in the pathogenesis of amyotrophic lateral sclerosis

研究課題名（英文）Axonal local translation and its implications in the pathogenesis of amyotrophic lateral sclerosis

研究代表者

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交付決定額（研究期間全体）：（直接経費）　　3,300,000円

研究成果の概要（和文）：私達の研究の目的は、ALS 患者のヒト多能性蒸気細胞（hIPSC）から運動ニューロン(MN)を抽出することで、ALSの進行を理解することである。主な成果は次の通り。
1．ALS患者hIPSC由来のMNの軸索生物学研究のためのマイクロ流体チャンバーの開発。2．hIPSC からのMN分化収率を 70% 以上に高める。3．ALSの病理を再現し、ALS MN培養物における軸索変性ミトコンドリア機能不全、及びタンパク質凝集を観察する。4．細胞内 ATPレベルの低下は、ALS MNにおけるTDP43凝集の増加を引き起こす。5．継続的な検証により、培地から潜在的なALSバイオマーカーを特定する。

研究成果の学術的意義や社会的意義
ALS rapidly leads to death. Our research links mitochondrial defects and reduction of ATP, to cytoplasmic aggregates in motor neurons from ALS patients, expanding on the role of LPS in disease. We also identified possible biomarkers for diagnosis, to improve patients` treatment and quality of life.

研究成果の概要（英文）：Our research aimed to understand ALS progression by deriving motor neurons (MNs) from ALS patient human pluripotent steam cells (hIPSCs), overcoming murine model limitations. Key achievements include:
1. Developing microfluidic chambers for axonal biology studies of MNs derived from ALS patients hIPSCs. 2. Enhancing MN differentiation yield from hIPSCs to over 70%. 3. Recapitulating ALS pathology, observing axonal degeneration, mitochondrial dysfunction, and protein aggregation in ALS MN cultures. 4. Identifying a mechanisms linking decrease in intracellular ATP levels to an increase of toxic TDP43 aggregation in ALS MNs. 5. Identifying potential ALS biomarkers from culture medium, with ongoing validation.

研究分野：Molecular Biology

キーワード：ALS　motor neurons　LPS　biomarkers　hIPSC

１．研究開始当初の背景（background of the project）

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder marked by the degeneration of upper and lower motor neurons (MNs). ALS leads to severe weakness and death from respiratory failure within 3-5 years. The rate of mortality within 12 months of diagnosis is 50% (Brown et al., 2017). The lifetime risk of ALS is 1 in 300, but the prevalence remains low because the prognosis is bleak. To date, ALS remains fatal with no cure, and the underlying disease mechanisms continue to elude our understanding. There remains an urgent need for an effective treatment to slow or to arrest the progression in ALS. Most research on ALS is conducted on animal models, which might not be a good surrogate of the human pathology, given the failure of just over 280 clinical trials in humans based on data generated by said models (Brown et al., 2017). To overcome the limitations of murine models we derived MNs from ALS patient's human pluripotent stem cells (hIPSCs) as the standpoint to shed light on the biological mechanisms of ALS and infer potential biological clues to improve diagnostics (e.g. biomarkers) and therapeutics. The discovery of new biomarkers will help in the diagnosis and treatment of the disease. Indeed, ALS is diagnosed through a combination of clinical assessments, medical history evaluations, and various tests, which eliminate other medical conditions with similar symptoms. Consequently, the diagnostic timeline for ALS can extend from several months to over a year. Delayed diagnosis, occurring late in the disease progression, critically limits the window for implementing supportive care and therapeutic interventions.

２．研究の目的（purpose of the project）

Our research concentrated on establishing a model for deriving motor neurons from ALS patients and identifying biomarkers for ALS. By generating motor neurons from hiPSCs, we can study the disease in a controlled environment, allowing for the identification of biomarkers, molecular indicators of disease presence or progression. Biomarkers that can reflect the underlying pathophysiology, disease progression, and response to therapies are vital for advancing ALS research and developing targeted treatments. Identifying reliable biomarkers can potentially expedite drug development, improve patient care, and provide a deeper understanding of ALS, ultimately enhancing the prospects for effective interventions and improved outcomes for individuals affected by this debilitating condition. Current biomarker discovery is hindered by the low levels of relevant proteins in accessible biofluids like blood. The use of hiPSCs to derive motor neurons represents a shift towards more ethical research practices by reducing reliance on animal models and providing a more relevant human context for studying the disease. It also allows for a closer look at these proteins without interference from the immune system.

In addition, we wanted to further our understanding of the mechanisms underlying ALS, by comparing a cohort of MNs generated by hIPSC for ALS patients with the most common mutations associated with the disease, including a sporadic line. We hope that not focusing on one mutation alone will uncover common mechanisms underlying the disease progression. We choose to focus on mitochondria and protein aggregation due to previous literature regarding mitochondrial defects in ALS (Genin EC et al, 2023) and our data suggesting a link between ATP production and liquid-liquid phase separation, which has been suggested to be contribute to protein aggregation.

３．研究の方法（research method）

Our research relied on the differentiation of MNs from hIPSCs. We first adopted a differentiation protocol based on Bossolasco et al., 2018, which gave us a relative low yield of MNs. We then adopted a different protocol based on Du et al., 2015, which yield a much higher percentage of MNs (>70%).

We produced microfluidics chamber for the purpose of validation of axonal transport defects as well as assessing axonal local translation in MNs from ALS patients. MFCs were designed and fabricated as described in Emily M.F. et al, 2022.

Protein liquid phase separation in ALS MNs was assessed by confocal microscopy following fluorescence recovery after photobleaching (FRAP) of axonal cytosolic GFP transduced via AVV vectors. Mitochondrial activity and fucntion was monitored by imaging using fluorescent membrane potential sensor TMRE or the Agilent Seahorse Cell Mito Stress Test kit in

combination with the Seahorse XF Analizer. Axonal ATP concentration was quantified by *in vitro* bioluminescence assay.
Proteomics analysis of tissue culture media was performed in the proteomics facility at OIST as described in El-Agamy SE et al, 2023. The mass spectrometer used was a Orbitrap Fusion Lumos from Themo Fisher. Quantification of the relative protein abundance was performed with the Proteome Dicoverer software suit and R.

４．研究成果

The project was about understanding what drives the rate of progression of neurodegeneration, looking at a paradigm of fatal neurodegenerative disorders: Amyotrophic Lateral Sclerosis (ALS). To overcome the limitations of murine models, which recapitulate the most salient features of the human disease but are not a complete surrogate of the human pathology, we directly derived motor neurons (MNs) from ALS patient hIPSC to assess underlying pathological mechanisms and to identify new biomarkers of disease. Below is a synopsis of our research achievements.

Establishment of compartmentalized cultures for ALS motor neurons. We have developed a microfluidics chamber (MFC) device suitable for the culture of MNs and adult sensory neurons. These MFCs rely on fluidic separation of the somatic and axonal compartments, allowing for the study of axonal biology, long-range intracellular transport and local translation via incorporation of puromycin to follow newly translated proteins via imaging as well as by biochemical retrieval and Mass Spectrometry analysis (Emily M.F. et al, 2022).

Motor neuron differentiation from human iPSCs. We acquired 6 ALS and 4 healthy control hiPSC lines from Cedar Sinai Cell Bank. These cells we specifically selected to cover the most common mutations linked to ALS, including sporadic cases. We have also perfected our differentiation protocol to significantly increase the yield of MNs differentiation from approximately 50% (following protocol from Bossolasco et al., 2018) to >70% (following protocol from Du et al., 2015). The percentage of Hb9 and ChAT positive neurons in our culture was examined and quantified by fluorescence confocal microscopy in various iPSC-derived motor neuron cultures and we now routinely obtained higher differentiation yield under the modified protocol from Du et al.

Characterization of pathogenic mechanisms in human iPSC-derived motor neuron culture. We assessed whether our motor neuron cultures could recapitulate the pathological characteristics of ALS observed in patients such as axonal degeneration, mitochondrial disfunction, and protein aggregation. We observed an increase in axonal blebbing in ALS MNs compared to healthy control cultures from day *in vitro* (div) 15. Significant increase in axonal degeneration was noticed in ALS motor neuron cultures after div30. To assess mitochondrial disfunction we performed in vitro bioluminescence assay to measure the intracellular concentration of ATP in all our iPSC-derived motor neuron cultures. We observed a significant reduction of ATP from TDP43 and C9Orf72 mutant ALS neurons compared to healthy control neurons, while ATP remained apparently stable in SOD1 and sporadic ALS neurons. In addition, we performed measurement and quantification of mitochondria number, dynamics and function, which have revealed an alteration in mitochondrial function for most of the lines.

Fluidic phase separation and ATP deficits in ALS MNs. A common feature of neurodegenerative disorders is the formation of protein aggregates by liquid-liquid phase separation (LLPS) (Ross and Poirier, 2004; Elbaum-Garfinkle, 2019), where proteins condensate to form membrane-less organelles. LPS has recently emerged as an essential mechanism to regulate the subcellular organization and function of synapses in physiological and pathological conditions (Chen et al., 2020). TAR-DNA-Binding protein 43 (TDP-43) is a component of pathological intracellular aggregates observed in most forms of ALS (Scotter et al., 2015), and its presence in RNA and/or stress granules has been reported to rely on LPS (Gopal et al., 2017; Tsang et al., 2019). We correlated lower levels of intracellular axonal ATP to an increase density of the axonal cytoplasm in ALS MNs compared to healthy controls. We speculated a correlation with the cytoplasm density and liquid phase separation, a phenomenon, which drives protein aggregation. Indeed, we have also assessed TDP43 aggregation in ALS MNs at different time points and in different culture condition. Preliminary observations suggest that TDP43 aggregation in soma and axon compartments might be occurring from div7. Analysis of TD43 aggregation is ongoing at later stages of

culture (div 14, div21 and div35) for all the lines.

Identification and characterization of novel ALS biomarkers from culture medium. We collected culture medium from ALS motor neuron cultures at 4 different time: div0, div7 (pre-symptomatic), div28 (early onset of disease) and div42 (late stage of disease). The medium has been subjected to proteomics analysis. Possible biomarkers have been identified by bioinformatic analysis, which compares disease progression in each individual ALS MN line as well as comparing ALS patient lines vs healthy controls. We are in the process of validating candidates in our culture system as well as in patient samples.

Conclusions: In summary, we have developed microfluidic chambers for culturing ALS motor neurons (MNs) and adult sensory neurons, enabling detailed studies of axonal biology and protein translation. Our refined protocol for differentiating MNs from hiPSCs has significantly increased yield, enhancing our experimental throughput. Our human iPSC-derived MN cultures successfully recapitulate key pathological features of ALS, including axonal degeneration, mitochondrial dysfunction, and protein aggregation. We observed notable axonal blebbing and degeneration in ALS MNs, particularly from day in vitro (div) 15 to div30. Mitochondrial dysfunction, evidenced by reduced ATP levels, was particularly pronounced in TDP43 and C9Orf72 mutant neurons. We have also explored the role of liquid phase separation (LPS) in ALS pathology, correlating reduced intracellular ATP levels with increased cytoplasmic density and TDP43 aggregation. Preliminary data indicates TDP43 aggregation as early as div7, with ongoing analysis at later stages.
Furthermore, our proteomic analysis of culture medium at various stages of disease progression has identified potential ALS biomarkers, which are currently being validated. These achievements not only deepen our understanding of ALS pathogenesis but also pave the way for developing new therapeutic strategies and diagnostic tools. Our finding connecting ATP cytosolic levels with toxic protein aggregation could be leveraged to explore chemicals increasing local ATP concentration via activation of mitochondrial activity. We will also validate the newly identified biomarkers in patient samples and strive to bring these findings to the clinic.

５．主な発表論文等

〔雑誌論文〕　計4件（うち査読付論文　1件／うち国際共著　2件／うちオープンアクセス　1件）

１．著者名 El-Agamy Sara Emad、Guillaud Laurent、Kono Keiko、Wu Yibo、Terenzio Marco	４．巻 22
２．論文標題 FMRP Long-Range Transport and Degradation Are Mediated by Dynlrb1 in Sensory Neurons	５．発行年 2023年
３．雑誌名 Molecular & Cellular Proteomics	６．最初と最後の頁 100653～100653
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.mcpro.2023.100653	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

１．著者名 Agrawal L, Vimal SK, Barzaghi P, Shiga T, Terenzio M.	４．巻 22
２．論文標題 Biodegradable and Electrically Conductive Melanin-Poly (3-Hydroxybutyrate) 3D Fibrous Scaffolds for Neural Tissue Engineering Applications	５．発行年 2022年
３．雑誌名 Macromol Biosci	６．最初と最後の頁 n/a - n/a
掲載論文のDOI（デジタルオブジェクト識別子） 10.1002/mabi.202200315	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 L. Agrawal, M. Saidani, L. Guillaud, M. Terenzio	４．巻 131
２．論文標題 Development of 3D culture scaffolds for directional neuronal growth using 2-photon lithography	５．発行年 2021年
３．雑誌名 Materials Science and Engineering	６．最初と最後の頁 -
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.msec.2021.112502	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Guillaud Laurent、El-Agamy Sara Emad、Otsuki Miki、Terenzio Marco	４．巻 13
２．論文標題 Anterograde Axonal Transport in Neuronal Homeostasis and Disease	５．発行年 2020年
３．雑誌名 Frontiers in Molecular Neuroscience	６．最初と最後の頁 N/A
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fnmol.2020.556175	査読の有無 無
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 －

〔学会発表〕　計14件（うち招待講演　13件／うち国際学会　14件）

1．発表者名
Marco Terenzio

2．発表標題
FMRP axonal retrograde transport is critical for its clearance and regulation of translational activity

3．学会等名
KGRI Lecture Series: Seminar on ALS, Department of Physiology, School of Medicine, Keio University, Tokyo, Japan.（招待講演）（国際学会）

4．発表年
2023年

1．発表者名
Marco Terenzio

2．発表標題
FMRP long-range transport and degradation are mediated by Dynlrb1 in sensory neurons

3．学会等名
The 46th annual meeting of the Japan Neuroscience Society. Sendai, Japan.（招待講演）（国際学会）

4．発表年
2023年

1．発表者名
Marco Terenzio

2．発表標題
Investigating axonal molecular dynamics in regeneration and neurodegenerative diseases

3．学会等名
Center for Integrative Biology, University of Trento, Trento, Italy.（招待講演）（国際学会）

4．発表年
2024年

1．発表者名
Marco Terenzio

2．発表標題
“FMRP long-range transport and degradation are mediated by Dynlrb1 in sensory neurons”

3．学会等名
46th annual meeting of the Japan Neuroscience Society. Sendai, Japan（招待講演）（国際学会）

4．発表年
2023年

１．発表者名
Marco Terenzio

２．発表標題
“FMRP axonal retrograde transport is critical for its clearance and regulation of translational activity”

３．学会等名
KGRI Lecture Series: Seminar on ALS, Department of Physiology, School of Medicine, Keio University, Tokyo, Japan（招待講演）（国際学会）

４．発表年
2023年

１．発表者名
Marco Terenzio

２．発表標題
“Regulation of RNP granule dynamics and axonal translation in sensory and motor neurons”

３．学会等名
OIST-RIKEN meeting, OIST, Japan（招待講演）（国際学会）

４．発表年
2023年

１．発表者名
Marco Terenzio

２．発表標題
“Investigating axonal molecular dynamics in regeneration and neurodegenerative diseases”

３．学会等名
invited talk at the Center for Integrative Biology, University of Trento, Trento, Italy（招待講演）（国際学会）

４．発表年
2024年

１．発表者名
Marco Terenzio

２．発表標題
“Dynein Roadblock 1 mediates axonal transport and degradation of FMRP1 in sensory neurons”.

３．学会等名
“Mechanisms of neuronal remodelling” EMBO workshop（招待講演）（国際学会）

４．発表年
2023年

１．発表者名
Marco Terenzio

２．発表標題
“Dynein-FMRP interaction is critical for FMRP granules motility and clearance in neurons”

３．学会等名
Ajou University School of Medicine (Korea) - OIST Joint Seminar（招待講演） （国際学会）

４．発表年
2023年

１．発表者名
Marco Terenzio

２．発表標題
“Dynein Roadblock 1 mediates axonal transport and degradation of FMRP1 in sensory neurons

３．学会等名
Invited talk via Zoom at Turin University, Italy（招待講演） （国際学会）

４．発表年
2022年

１．発表者名
Marco Terenzio

２．発表標題
“Dynein dynamics and signaling in neuronal survival and regeneration”

３．学会等名
ADR2022（国際学会）

４．発表年
2022年

１．発表者名
Laurent Guillaud

２．発表標題
Hydrotropic regulation of neuronal proteins aggregation in synaptic organization and neurodegeneration

３．学会等名
ADR2021 workshop (https://groups.oist.jp/adr/program)（招待講演） （国際学会）

４．発表年
2021年

１．発表者名 Marco Terenzio
２．発表標題 Essential Role of Dyne Road Block1 in the survival of adult sensory neurons
３．学会等名 7th East Asia Joint Symposium (https://www.ims.u-tokyo.ac.jp/imsut/en/EAJS2021.html)（招待講演）（国際学会）
４．発表年 2021年

１．発表者名 Marco Terenzio
２．発表標題 Investigating axonal molecular dynamics in regeneration and neurodegenerative diseases
３．学会等名 Max Planck Institute for Polymer Research in Germany（招待講演）（国際学会）
４．発表年 2022年

〔図書〕　計1件

１．著者名 Emily Maria Fransiska, Agrawal Lokesh, Barzaghi Paolo. Otsuki Miki, Terenzio Marco	４．発行年 2022年
２．出版社 Humana	５．総ページ数 588
３．書名 Axonal Transport	

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関