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研究課題名（和文）Drug screening system for early pathology of SBMA using disease specific iPSCs and novel biomarkers

研究課題名（英文）Drug screening system for early pathology of SBMA using disease specific iPSCs and novel biomarkers

研究代表者

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研究成果の概要（和文）：球脊髄性筋萎縮症(SBMA)はアンドロゲン受容体の変異により発症する、運動神経変性疾患である。現在、有効な治療法がない難治性疾患の一つである。近年、人工多能性幹細胞(iPSC)を利用した疾患モデルが可能になり、我々の研究グループはiPSCを利用してSBMAモデルの作製を進めてきた。本研究では、iPS細胞由来運動ニューロンの培養条件を最適化することでSBMAの病態をより明確に検出し得る培養条件を見出した。さらに、SBMAの発症に関わる分子メカニズムを検討した。本研究で得られた結果により、新たな治療標的の探索や新規治療薬スクリーニングが期待される。

研究成果の学術的意義や社会的意義
Currently there is no effective treatment for SBMA. Our research has perfected an iPSC model of SBMA and clarified molecular mechanisms causing the disease, allowing for faster screening of potential drugs for its treatment, and better understanding of the intracellular causes of the disease.

研究成果の概要（英文）：Spinal and bulbar muscular atrophy (SBMA) is an adult-onset slowly progressive motor neuron (MN) disease caused by the expansion of CAG repeat in the Androgen Receptor (AR) gene, for which current treatments are ineffective. Induced Pluripotent Stem Cells (iPSCs) are a valuable tool for developing reproducible models of diseases, and we succeeded in developing a model of SBMA using iPSC-derived MNs. In this project we developed an optimized culture system incorporating stressors that facilitates the observation of the SBMA in cultured MNs, clarified the molecular mechanisms contributing to disease onset and progression and developed appropriate isogenic control cell lines, facilitating comparisons and allowing a clearer observation of phenotypes. These results allow us to more clearly investigate the etiology of SBMA and test novel potentially life-saving treatments.

研究分野：Neuroscience

キーワード：Motor Neurons　SBMA　Neurodegeneration　iPSC

１．研究開始当初の背景

Spinal and bulbar muscular atrophy (SBMA) is an adult onset, slowly progressive motor neuron disease. Analyses of SBMA model mice have revealed a mutant androgen receptor (AR) with an expanded polyglutamine tract (CAG repeats) (La Spada et al., Nature, 1991) which forms aggregates in a ligand (testosterone)-dependent manner, causing motor neuron degeneration via transcriptional dysregulation, abnormal intracellular signal transduction and axonal transport impairments.

Studies have shown that treatment with an anti-androgen, Leuprorelin, is effective only in patients in the early stages of the disease (Katsuno et al., The Lancet Neurology, 2010), indicating that it is necessary to clarify the early pathology of SBMA and develop better methods to track disease onset and progression. Our previous study has shown that motor neurons derived from SBMA patient-derived induced pluripotent stem cells (iPSCs) did not exhibit mutant AR aggregation, but could recapitulate early pathology of SBMA such as decreased neurite extension, increased expression of CALCA, and increased c-Jun phosphorylation, suggesting these patients-derived motor neurons could be an early disease model of SBMA before the formation of AR aggregates.

Using this model, we identified four pathogenic factors upregulated in patient-derived motor neurons from the early stage of the disease. Our preliminary data has shown that addition of these four factors induces SBMA pathology in control iPSC-derived motor neurons (Onodera et al., in preparation)

Our preliminary data also shows that cellular stress plays a major role in the progression of SBMA, as the application of specific stress factors causes a reduction in neurite length and an increase in the expression of stress markers, indicating that the motor neurons of SBMA patients are particularly vulnerable to cellular stress. This suggests that stress plays a large role in the progression of SBMA, and that there is a need to clarify factors contributing to the pathology of SBMA. Succeeding in identifying the molecular pathways responsible opens new potential avenues for pharmacological intervention in SBMA.

２．研究の目的

1) Establish a drug screening system for SBMA:

In this study we aimed to develop a high-throughput drug screening system that would allow for objective evaluation of several parameters to track the early pathology of SBMA. Systematic evaluation of these parameters would aid in establishing causal relationships and correlations between them, helping identify the molecular causes of SBMA. We initially aimed to use this system to test a library of existing drugs and drugs of known function, with a potential for the discovery and screening of compounds that could be used to treat SBMA in the near future.

2) Validate the use of biomarkers in SBMA:

Studies carried out by our group have identified four early pathogenic factors whose expression correlates with disease progression in experimental models. Clarifying the changes in intracellular signaling pathways evoked by these factors would help us understand the early phenomena occurring in SBMA, as well as validating their usability as biomarkers for SBMA, generating easily measurable parameters which could be used to evaluate the efficacy of novel treatments for SBMA.

3) Establish isogenic control iPSC lines:

Although patient-derived iPSCs can be utilized to develop experimental models that can be used to test possible treatments, the considerable differences in genetic backgrounds among iPSC clones (clonal variations) introduce several variables that affect the significance and reproducibility of findings. Therefore, we aimed to overcome this limitation by using gene editing to generate isogenic control iPSCs for studying the molecular mechanisms of SBMA.

３．研究の方法

1) Establish a drug screening system using SBMA motor neurons:

To standardize and clarify the differences between SBMA and control iPSC-derived motor neurons for drug screening, progression of SBMA phenotypes was evaluated using an automated imaging cytometer, where neurite extension was measured as a means of evaluating disease onset and progression in SBMA motor neurons. Concomitantly, we carried

out culture optimization in order to allow for better observation of SBMA phenotypes. Optimization of culture conditions was carried out by systematic reduction and/or withdrawal of neurotrophic factors, and the optimal combination where a marked difference in phenotype between healthy motor neurons and SBMA motor neurons could be observed was elected as the optimal basal culture condition.
Our preliminary studies have also shown stress inducers are capable of reducing neurite extension. To further optimize culture conditions and clarify the role of stressors in the onset and progression of SBMA, stress factors at different concentrations were added to optimized culture conditions. The combination and concentration of stress factors that induced the most marked difference between healthy motor neurons and SBMA motor neurons when added to the optimal basal medium was then utilized for subsequent experiments and evaluations.

2) Validate the usability of biomarkers for tracking of disease progression in SBMA:
Preliminary data has shown that motor neurons derived from SBMA iPSCs display impaired neurite extension and higher levels of phosphorylated c-Jun. Further experimentation has shown a similar phenotype can be observed when motor neurons derived from control iPSCs are treated with the aforementioned four pathogenic factors. We verified the usability of these factors as biomarkers in SBMA and indicators of disease progression by quantifying their levels in motor neurons derived from SBMA and control iPSCs, reducing their expression with lentiviral shRNA and evaluating the effects of their overexpression. The pathways involved were evaluated by investigating the phosphorylation of downstream molecules of the four factors by western blotting. We will also assess the expressions of four factors in patients' spinal cord tissue and serum.

3) Establishment of isogenic control iPSCs:
Cell-based models for research on the causes of SBMA rely on comparing observable pathology in patient-derived iPSCs to control iPSCs. The main limitations of this model are the considerable genetic differences that severely hinder the reproducibility of results and the acquisition of reliable, significant data. We aimed to overcome this limitation by developing an isogenic control-iPSC line from SBMA iPSCs, allowing for a more apt comparison that minimizes or eliminates other confounding factors. This was achieved by using a PiggyBac footprint-free system on SBMA patient-derived iPSCs in order to shorten the CAG repeats of AR to a length observed in healthy controls.

４．研究成果

1) Establish a drug screening system using SBMA motor neurons:
Among all culture conditions tested in this study, we observed that a reduction and/or withdrawal of some of the trophic factors caused marked neurite reduction in SBMA motor neurons, while still being able to support normal neurite extension in healthy motor neurons. Furthermore, we observed that these optimized conditions allowed the observation of the SBMA phenotype, as measured by neurite degeneration, as early as 6 weeks in culture, whereas such a difference would require 8 weeks of culture under previous unoptimized conditions. Therefore, by reducing trophic factors, SBMA iPSC-derived motor neurons could show earlier and more pronounced pathological changes, which facilitates faster screening.
Furthermore, we succeeded in demonstrating that the addition of a stress factor in the culture was able to further hasten the onset of SBMA, with marked neurite reduction occurring as early as 3 weeks in culture, while causing no discernible impairment to healthy motor neurons. These results suggest that an optimized culture condition that incorporates stressors and lower supplementation allows for easier, more reproducible observation of SBMA in cultured neurons. Furthermore, developing upon the roles these factors play in neuronal development could assist in clarifying the etiology of SBMA.
We succeeded in identifying the optimal conditions for observing SBMA pathology in cultured motor neurons, and the screening of drugs that could slow or revert this phenotype will be the subject of a future follow-up study.

2) Validate the use of biomarkers in SBMA:
Upon the identification of four pathogenic factors that contributed to the etiology of SBMA, we proceeded to verify the mechanisms through which they contributed to observed pathology. To elucidate downstream signaling pathways of those four pathogenic factors, we performed Western blot analysis of the samples obtained from control motor neurons treated with each of the four pathogenic factors for several phosphorylated signal proteins. The obtained data showed alteration of some of the signals, including theJNK/c-Jun pathway, that could induce

cytotoxicity and apoptosis. These data suggest that these signaling pathways may disrupt normal cellular functions or trigger pathological processes, and could be potential therapeutic targets.
We also proceeded to evaluate whether these four factors could be quantifiably measurable in samples obtained from patients as a means of validating their usability as biomarkers. We first performed immunohistochemical analysis of postmortem spinal cords of the affected patients for the expressions of the four pathogenic factors. As a results, the expressions of some of the four factors seemed to be altered in patients' motor neurons, but these results need to be further examined more in detail. Further studies are needed to validate whether or not these factors can be used as biomarkers in human patients.

3) Establish isogenic iPSC lines:
We succeeded in carrying out gene editing through the use of a footprint-free PiggyBac system, and obtained several gene edited clones derived from SBMA patient derived-iPSCs. The established edited iPSC clones were confirmed to have shortened (normal) CAG repeat of the AR gene, and were capable of differentiating into motor neurons appropriately. As of the present moment however, we have not been able to fully characterize these cells and confirm that the editing was successful in reversing the deleterious effects caused by abnormally long CAG repeats. Further studies will be carried out to characterize these cells and observe the effects of editing on the presentation of the SBMA phenotype.

５．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　0件／うち国際共著　0件／うちオープンアクセス　0件）

１．著者名 Onodera Kazunari、Shimojo Daisuke、Ishihara Yasuharu、Yano Masato、Miya Fuyuki、Banno Haruhiko、Kuzumaki Naoko、Ito Takuji、Okada Rina、de Araujo Herculano Bruno、Ohyama Manabu、Yoshida Mari、Tsunoda Tatsuhiko、Katsuno Masahisa、Doyu Manabu、Sobue Gen、Okano Hideyuki、Okada Yohei	４．巻 13
２．論文標題 Unveiling synapse pathology in spinal bulbar muscular atrophy by genome-wide transcriptome analysis of purified motor neurons derived from disease specific iPSCs	５．発行年 2020年
３．雑誌名 Molecular Brain	６．最初と最後の頁 18
掲載論文のDOI（デジタルオブジェクト識別子） 10.1186/s13041-020-0561-1	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4件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 エルクラノ ブルーノ、小野寺一成、伊藤卓治、下門大祐、岡田梨奈、勝野雅央、道勇学、祖父江元、岡野栄之、岡田洋平
２．発表標題 Drug screening for early pathology of SBMA using disease specific iPSCs and novel biomarkers.
３．学会等名 第19回日本再生医療学会総会(JSRM)
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４．発表年 2021年

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３．学会等名 ISSCR/JSRM 2021 Tokyo International Symposium
４．発表年 2021年

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２．発表標題 Elucidating early pathophysiology of spinal-bulbar muscular atrophy using disease-specific iPSCs
３．学会等名 第63回日本神経学会学術大会
４．発表年 2022年

〔図書〕　計0件

〔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