論文の内容の要旨

A Patient-derived iPSC Model Revealed the Involvement of Oxidative Stress in Molecular Pathogenesis of Facio-scapulo-humeral Muscular Dystrophy (FSHD)

（ 患者由来 iPS 細胞モデルで明らかとなった顔面肩甲上腕型筋ジストロフィ(FSHD)における酸化ストレスの病態関与 ）

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The muscular dystrophies are a group of inherited myogenic diseases that cause progressive skeletal muscle wasting and weakness, and consist of varieties of types with distinct genetic backgrounds and clinical features. In this thesis, I focus on facio-scapulo-humeral muscular dystrophy (FSHD), which is a type of muscular dystrophy strongly associated with epigenetic dysregulation, and aimed at elucidating its pathology.

FSHD is named after muscle areas that are likely to be initially affected, but as symptoms progress, the disease can affect muscles in the whole body, and in some cases patients are forced to use wheel chairs. FSHD patients show unique characteristics of muscle weakness as compared to other types of muscular dystrophies: relatively late onsets of disease phenotypes (typically during the second decade), asymmetric patterns of muscle weakness, and large variations in disease progression among patients. These characteriscs implicate that there are major environmental factors that regulate disease onset and progression even though it is a genetic disease.

DUX4, which is considered as the causative gene of FSHD, is silenced in most of somatic cells in healthy individuals, but is ectopically expressed in the skeletal muscle cells of FSHD patients because of aberrant chromatin relaxation at sub-telomeric region 4q35. The genome at 4q35 in healthy individuals normally contains more than 10 sequential repeat units called D4Z4 which consists of 3.3kp per unit, and forms heterochromatin characterized by DNA hyper-methylation and accumulation of histone 3 lysine 9 tri-methylation (H3K9me3). The diminished heterochromatic state at 4q35 in FSHD patients is caused by either reduced number of D4Z4 repeats (1-10 units) (patients with this case are classified as FSHD type 1, FSHD1) or mutations in genes encoding chromatin regulators, such as *SMCHD1* (structural maintenance of chromosomes flexible hinge domain

containing 1) (patients with this case are classified as FSHD type 2, FSHD2) (Figure.1). However, the mechanism by which *DUX4* expression is regulated remains largely unknown. Furthermore, the low-level expression of *DUX4* raises questions regarding its functional impact, because no report have detected of *DUX4* proteins in muscle biopsies in FSHD patients and because patient-derived primary cultured myoblasts showed low stochastic pattern and distinct levels of *DUX4* expression. In this thesis, I aimed at understanding the pathology in the terms of gene regulation of endogenous *DUX4* expression.

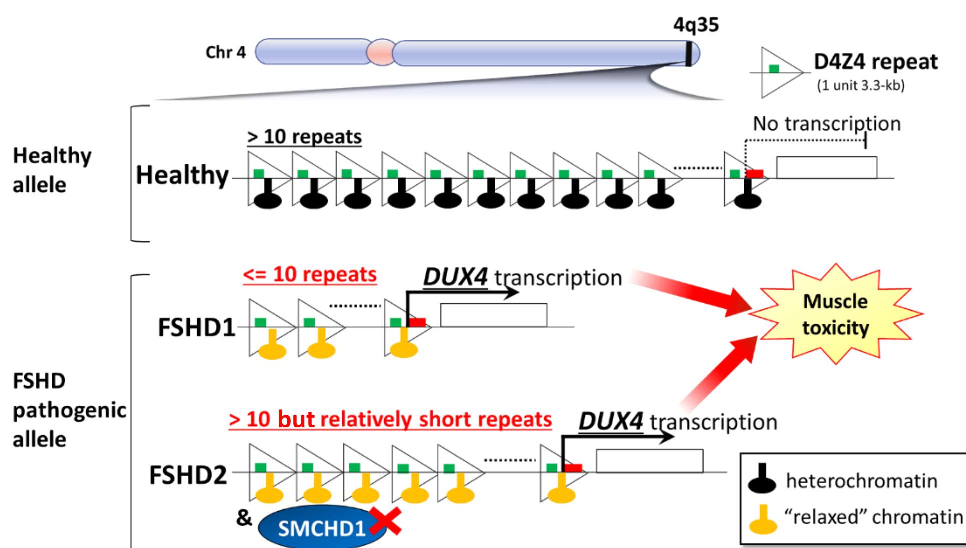

Figure.1 Scheme of genetic backgrounds of FSHD

Induced pluripotent stem cells (iPSCs) opened the door to utilize rare disease patient-derived cellular materials as a feasible and unlimited source for pathological investigation. As FSHD is caused by highly complex genetic backgrounds and the 4q35 genome structure is conserved only in a limited group of primates, patient-derived cells were suitable for FSHD study. Especially, iPSCs are supposed to be more suitable compared to patient-derived primary culture, because primary muscle cultures fundamentally have a limited cell cycle.

To analyze the endogenous *DUX4* expression in patient-derived muscle cells, I established a myocyte model developed from FSHD patient-derived iPSCs. First, iPSC clones were established from a FSHD1 patient (F1), a FSHD2 patient (F2), and a healthy individual (HC) at the laboratory of Dr. Hidetoshi Sakurai, at the Center for iPS Cell Research and Application, Kyoto University. Then by transiently overexpressing MyoD, a master regulator of myogenic linage, the iPSC clones were efficiently differentiated into myosin heavy chain (MyHC) positive myocytes (Figure.2A). Among these myocytes, F1-derived and F2-derived myocytes showed substantial mRNA expression of *DUX4* and its direct downstream targets of *DUX4* transcriptional activity including *ZSCAN4*, but not HC-derived myocytes (Figure.2B). To precisely evaluate the relationship between the genetic background and *DUX4* gene expression, I used genome-editing technology and generated gene-corrected FSHD2-derived clones (isogenic control clones, ICs) by replacing *SMCHD1* mutation with the wild type sequence in F2 iPSC clones. IC-derived myocytes showed suppressed expression of *DUX4* and *ZSCAN4* compared to parental F2-derived myocytes (Figure.2C). These data suggested that the established myocyte model is suitable for further studies of *DUX4* expression.

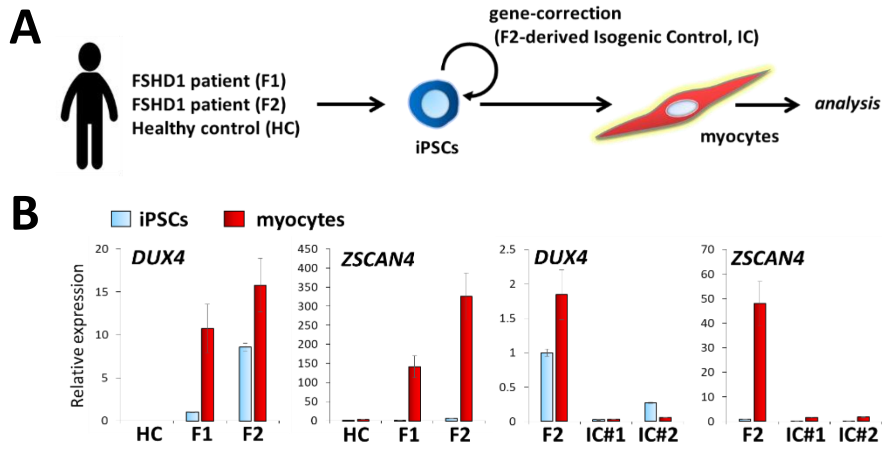

Figure.2 A) A schematic illustration of myocyte model developed from patient-derived iPSCs. B and C) Real-time quantitative PCR (RT-qPCR) analysis for *DUX4* and *ZSCAN4* among iPSCs and myocytes derived from Healthy Control (HC), FSHD1 (F1), FSHD2 (F2), gene-corrected FSHD2 isogenic control (IC#1, IC#2).

Next, I hypothesized that there are extracellular environmental factors that regulate disease progression by altering endogenous *DUX4* expression. As a possible candidate factor, I focus on oxidative stress (OS), which is a common environmental stress in skeletal muscle, because muscle biopsies in FSHD expressed increased markers of OS, and because a small clinical study showed that oral supplementation of antioxidants partially improved muscle function in FSHD patients. Thus, I investigated whether OS can alter *DUX4* expression. By stimulating myocytes of each clone with H_2O_2, which is a model of OS, we determined that *DUX4* expression was specifically increased by OS in both F1 and F2 myocytes (Figure.3). Moreover, gene-corrected IC-derived myocytes showed a marked suppression of OS-induced *DUX4* upregulation (Figure.3), demonstrating that OS-induced *DUX4* increase is a disease-specific molecular phenotype. By chromatin immunoprecipitation (ChIP) analysis, H3K9me3 and HP1γ (heterochromatin protein 1 gamma) were increased at 4q35 in IC-derived

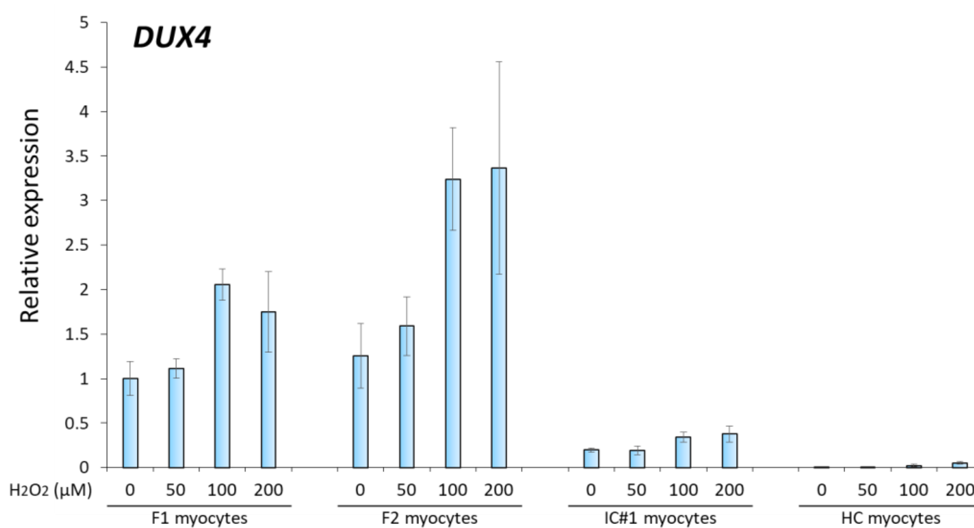

Figure.3 RT-qPCR analysis showed H_2O_2 stimulation induced *DUX4* increase in F1- and F2-derived myocytes and its suppression in gene-corrected IC- and HC-derived myocytes.

myocytes compared to parental F2-derived myocytes, indicating that diminished heterochromatic state in FSHD myocytes permits basal and OS-induced transcription. I further found that DNA damage response (DDR) was involved in OS-induced *DUX4* upregulation and identified ATM (ataxia-telangiectasia mutated kinase), a DDR regulator, as a mediator of this effect. These results suggest that the relaxed chromatin state in FSHD muscle cells permits aberrant access for OS-induced DDR signaling to increase *DUX4* expression (Figure.4).

In conclusion, this new model of FSHD muscle cells developed from patient-derived iPSCs revealed that OS upregulates FSHD-causative *DUX4* through aberrant access of DNA damage response signaling, which is a previously unrecognized mechanism by which *DUX4* expression is regulated in FSHD muscle cells. Thus, OS may represent an environmental risk factor that promotes FSHD progression through *DUX4* gene expression, and this may explain the phenotypic hallmarks of FSHD such as asymmetric patterns of muscle atrophy and varieties of disease progression. This new FSHD model should also provide a basis for drug development and discovery of therapeutic targets for FSHD.

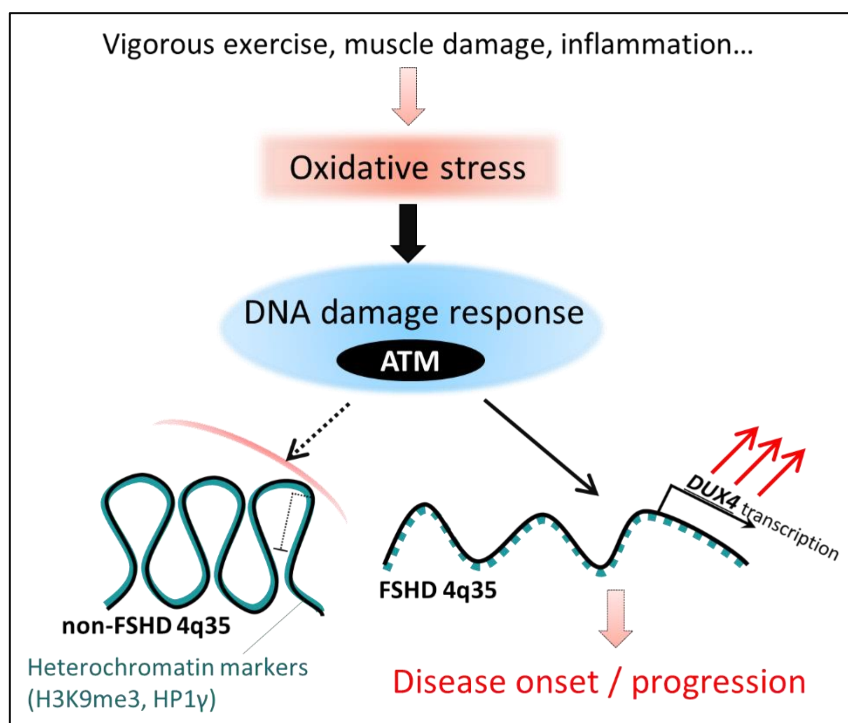

Figure.4 Proposed scheme of involvement of oxidative stress in FSHD pathology propose from this study.