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研究課題名（和文）Mechanistic basis of new generation laminin (NGL) for developing paraxial mesoderm cell lineages from hiPSCs

研究課題名（英文）Mechanistic basis of new generation laminin (NGL) for developing paraxial mesoderm cell lineages from hiPSCs

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研究成果の概要（和文）：申請者は所属研究室が確立した筋分化誘導プロトコールを利用して、NGL(次世代ラミニン)が動物由来のマトリゲルよりヒトiPS細胞(hiPSC)から骨格筋前駆細胞の誘導効率を増加することができ、さらに骨格筋細胞や骨格筋階細胞の誘導効率も上げることを判明した。本研究では、NGLがhiPSCからの筋分化を制御するメカニズムを解明した。NGLの独特構造であるヘパラン硫酸(HS)はFGF受容体を介するシグナル伝達を活性化することによって、筋細胞への分化を促進することがわかった。本研究で確立したhiPSCから骨格筋細胞、骨格筋幹細胞を誘導するシステムは疾患モデリングおよび臨床応用に有用であると考えられる。

研究成果の学術的意義や社会的意義
Using Xeno-free next generation laminin, we establish a highly efficient differentiation system from hiPSCs, providing a universal differentiation protocol for disease modeling and cell therapy. We also elucidate the role of FGFs in the next generation laminin inducing paraxial mesoderm from hiPSCs.

研究成果の概要（英文）：hiPSCs provide an attractive cell source of cell therapies and disease modeling for muscular dystrophy. We has established the protocol of generating muscle stem cells (MuSCs) and myocytes from hiPSCs. In this project, using next generation laminin (NGL, p421), we established more efficient protocol for generating myocytes and MuSCs. We revealed that the heparan sulfate chains (HS) in p421 regulate myogenic differentiation from hiPSCs, by regulating FGFR signaling pathway before paraxial mesoderm formation in the step-wised protocol. Utilizing the exogenous bFGF and endogenous FGFs, HS in p421 stimulated the phosphorylation of ERK in the downstream of FGFR, regulating the gene expression of primitive streak and paraxial mesoderm. In his project, we elucidated the mechanism of p421 regulating myogenic differentiation from hiPSCs and established a universal myogenic differentiation protocol for skeletal muscle disease modeling and cell therapy.

研究分野：幹細胞生物学

キーワード：次世代ラミニン　iPS細胞　骨格筋幹細胞　再生医学

１．研究開始当初の背景

Human induced pluripotent stem cells (hiPSCs) need to culture on suitable extracellular matrix (ECM). ECM is a dynamic and complex environment characterized by biochemical properties able to regulate stem cell differentiation. Laminins are a family of glycoproteins present in ECM. Laminin E8 fragments (LM-E8s), serving as a functionally minimal form of laminin, has been used as Xeno-free substrate for maintaining undifferentiated hiPSCs. However, LM-E8s provided ineffective hiPSCs differentiation to myocyte and muscle stem cells (MuSCs) in established protocol. Thereby, we designed next generation laminin (NGL) which has unique structure: perlecan D1 domain with three heparan sulfate chains (PlnD1-HS) conjugate to the C-terminus of Laminin E8 fragments. Heparan sulfate side chains (HS) are highly sulfated polysaccharide in ECM, that act as essential co-receptors with various factors, allows embryonic development and stem cell lineage-specific differentiation. By screening all of NGL isoforms (p111, p211, p332, p411, p421, p511, p521) which could be obtained, we revealed that p421 provided the most efficient myogenic differentiation. Using p421 we successfully established a more efficient differentiation system for myocytes and MuSCs inducted from 201B7 hiPSC line. Our previous research also showed that p421 significantly improved paraxial mesoderm (PM) development from hiPSCs by regulating FGFR signaling pathway.

However, the deeper mechanism of p421 regulating FGFR signaling pathway is still unclear. Also, we did not establish a universal protocol for hiPSCs-MuSCs for disease modeling and for cell therapy.

２．研究の目的

In this project, we were seeking to elucidate the mechanism of p421 regulating hiPSCs differentiation, elucidating the role of HS in PM differentiation in vitro, and establishing a highly efficient differentiation system of hiPSCs-MuSCs for disease modeling and clinical application.

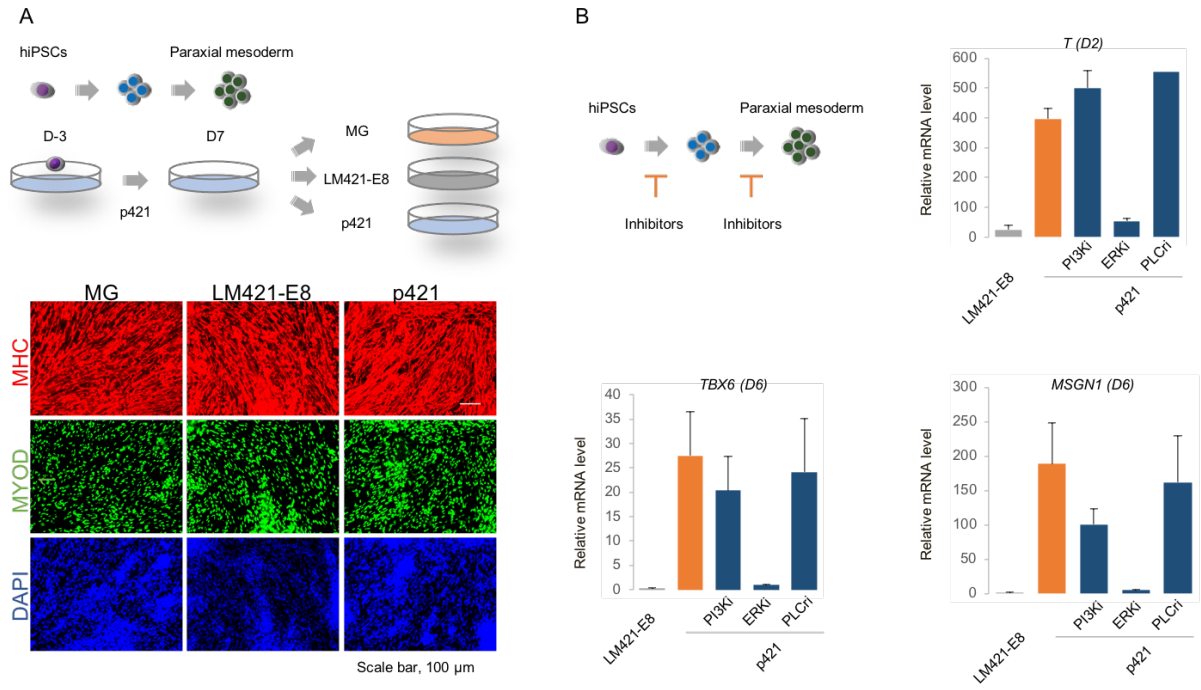
３．研究の方法

(1) In order to investigate the effects of HS in the downstream of FGFR, we inhibited the FGFR downstream phosphorylation of PI3K, ERK1/2 and PLCγ.
(2) Transcriptome analysis during hiPSCs differentiation was performed by RNA sequencing in step-wised differentiation stages.
(3) The effects of exogenous bFGF were confirmed by treating hiPSCs with mutated E96A and K125E bFGFs.
(4) By using Duchenne muscular dystrophy (DMD) and Miyoshi myopathy (MM) patients derived hiPSC lines, we established a universal protocol for myogenic differentiation from hiPSCs.

４．研究成果

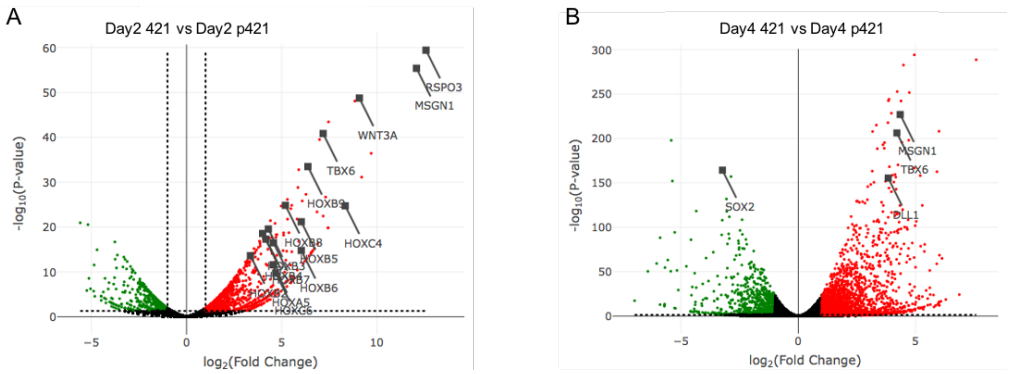
(1) In order to confirm the period of HS regulating FGFR signaling, we passaged PM cells which was cultured on p421 at Day 7 of differentiation to MG, LM421-E8 or p421. The immuno-staining of myosin heavy chain (MHC) and MYOD1 data showed that MG or LM421-E8 provided effective myogenic differentiation after cell passaging from p421, indicating that the effect of p421 is in the period before PM formation [Figure 1. A]. To investigate the effects of p421 in the downstream of FGFR, we treated hiPSCs with PI3K inhibitor (PIK90), ERK inhibitor (PD0325901) or PLCγ inhibitor (U73122) in the period of primitive streak formation or in the paraxial mesoderm formation [Figure 1. B]. ERK phosphorylation inhibitor remarkably decreased the marker genes expression of primitive streak or paraxial mesoderm, whereas PI3K inhibitor and PLCγ inhibitor had no effects, indicating that HS regulating FGFR through phosphorylation of ERK.

Figure 1. HS regulate FGFR pathway through phosphorylation of ERK before paraxial mesoderm formation



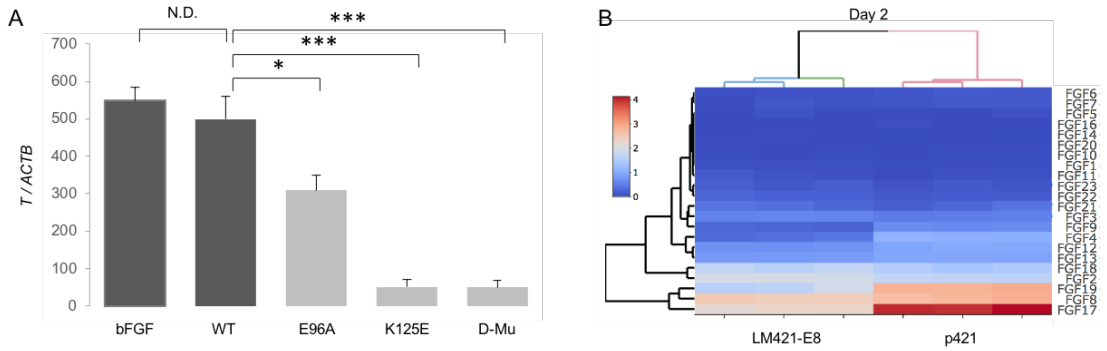
(2) The transcriptome analysis during hiPSCs differentiation showed that p421 significantly increased marker genes expression during primitive streak (Day2) and paraxial mesoderm formation (Day4). Comparing to p421, LM421-E8 highly expressed undifferentiated and neural markers (SOX2) on day 2 and day4, indicating the importance of FGF signaling in the primitive streak [Figure 2. A]and paraxial mesoderm formation [Figure 2. B].

Figure 2. p421 increased marker genes expression of primitive streak and paraxial mesoderm



(3) Since bFGF was not added into the stimulation medium from Day 0 of differentiation, we hypothesized that the bFGF in hiPSCs maintain medium (from Day -3 to Day 0) and endogenous FGFs secreted by differentiated cells has the paracrine function to regulate FGFR signaling. To confirm this hypothesis, we utilized the mutated bFGFs (E96A and K125E), which has the FGFR binding mutation. E96A, K125E or the double mutation in the hiPSCs maintain medium significantly decreased primitive streak genes expression on Day 2 of differentiation, indicating the effect of exogenous bFGF in primitive streak formation [Figure 3. A]. The transcriptome analysis by RNA sequencing indicated that the paracrine FGFs (FGF8, FGF17) were remarkable increased on Day 2 of differentiation, indicating the role of endogenous FGF in hiPSCs differentiation to primitive streak and paraxial mesoderm [Figure 3. B].

Figure 3. Exogenous and endogenous bFGF regulated myogenic differentiation from hiPSCs

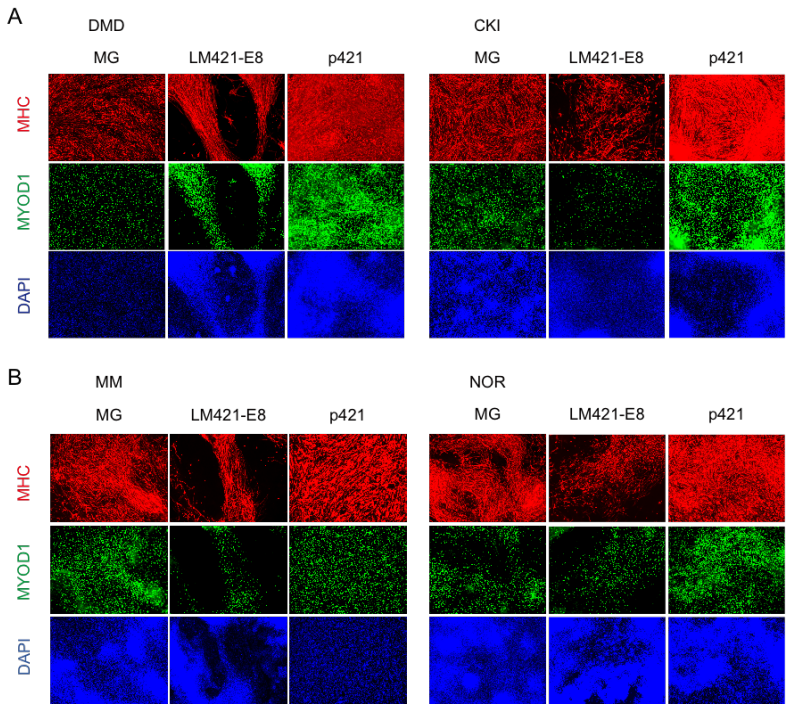


(4) Disease modeling and cell therapy need a universal differentiation protocol not only in the specific hiPSC line but also in patient derived hiPSC lines. Here we used DMD patient derived hiPSC lines and its gene corrected cell lines (CKI) to test the differentiation protocol. And also, we tested MM patient derived hiPSC line and its normal control cell line (NOR). P421 showed robust and stable myogenic differentiation in all these 4 cell lines comparing to in MG and LM421-E8 condition [Figure 4. A, B], suggesting the establishment of a universal method for patient derived cell by using this NGL.

Summary:

Muscular dystrophies are a group of rare muscle disorders that cause progressive muscle weakness and muscle degeneration. hiPSCs provide an attractive cell source for cell therapies and disease modeling. Our group has established a strategy for generating myocytes and MuSCs. However, this approach shows lower efficiency and requires MG which could not use for clinical application. Take advantage of NGL, we established a more efficient protocol for generating myocytes and MuSCs in several types of hiPSCs.

Figure 4. Establishing universal protocol of myogenic differentiation in hiPSCs



Moreover, we revealed that the HS in p421 regulate myogenic differentiation from hiPSCs, by regulating FGFR signaling pathway before paraxial mesoderm formation in the step-wised protocol. Utilizing the exogenous bFGF and endogenous FGFs, HS stimulated the phosphorylation of ERK in the downstream of FGFR, regulating the gene expression of primitive streak and paraxial mesoderm. P421 could provide more than 90% paraxial mesoderm cell population for the following differentiation (data not shown), which is beneficial in the following step of differentiation.

In conclusion, in this project we elucidated the mechanism of p421 regulating myogenic differentiation from hiPSCs and established a universal myogenic differentiation protocol for skeletal muscle disease modeling and cell therapy.

５．主な発表論文等

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

１．著者名 Zhao M, Tazumi A, Takayama S, Takenaka-Ninagawa N, Nalbandian M, Nagai M, Nakamura Y, Nakasa M, Watanabe A, Ikeya M, Hotta A, Ito Y, Sato T, and Sakurai H	４．巻 15
２．論文標題 Induced Fetal Human Muscle Stem Cells with High Therapeutic Potential in a Mouse Muscular Dystrophy Model	５．発行年 2020年
３．雑誌名 Stem Cell Reports	６．最初と最後の頁 89-94
掲載論文のDOI（デジタルオブジェクト識別子） 10.1016/j.stemcr.2020.06.004	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 －

〔学会発表〕　計5件（うち招待講演　0件／うち国際学会　3件）

１．発表者名 Mingming Zhao
２．発表標題 A structure-based extracellular matrix modification promotes paraxial mesoderm differentiation from human induced pluripotent stem cells
３．学会等名 Cira international Symposium 2019（国際学会）
４．発表年 2019年～2020年

１．発表者名 Mingming Zhao
２．発表標題 A STRUCTURE-BASED EXTRACELLULAR MATRIX MODIFICATION PROMOTES PARAXIAL MESODERM DIFFERENTIATION FROM HUMAN INDUCED PLURIPOTENT STEM CELLS
３．学会等名 International Society for Stem Cell Research, Annual meeting 2019（国際学会）
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３．学会等名 International Society for Stem Cell Research, Annual meeting 2020（国際学会）
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１．発表者名 Mingming Zhao
２．発表標題 EFFICIENT MYOGENIC DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS ON NEXT GENERATION LAMININ
３．学会等名 第6回日本筋学会学術集会
４．発表年 2020年～2021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