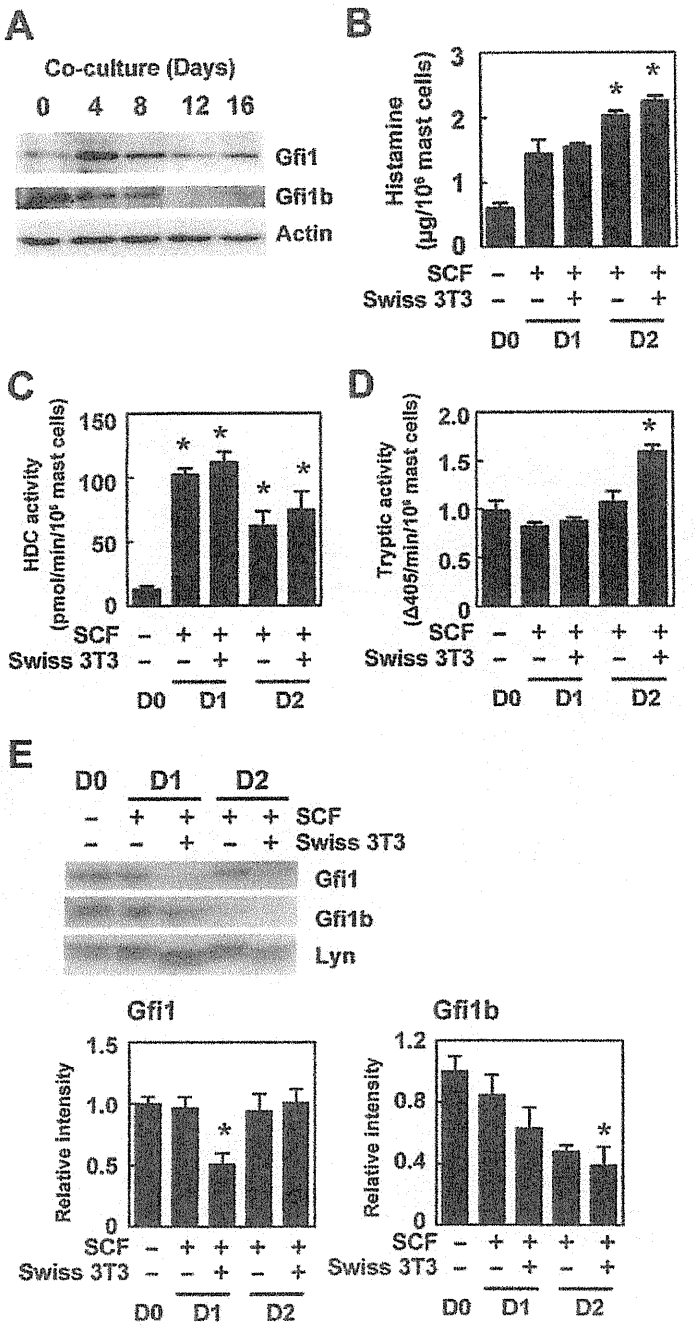

Fig. 1. Induction of Histamine Synthesis and Tryptase Expression in MC9 Cells by Co-culture with Swiss 3T3 Fibroblasts

(A) IL-3-dependent bone marrow-derived cultured mast cells were co-cultured with Swiss 3T3 fibroblasts in the presence of SCF for the periods indicated. Expression levels of Gfi1 and Gfi1b were determined by immunoblot analyses. Expression levels of β-actin were measured as the loading control. (B–D) MC9/mock cells were co-cultured with or without Swiss 3T3 fibroblasts in the presence of SCF (30 ng/mL) for 1 or 2 d. The cells were harvested and histamine content (B), HDC activity (C) and tryptic activity (D) were measured. The values were represented as the means±S.E.M. (n=3). (E) Expression levels of Gfi1 and Gfi1b in the cells were determined by immunoblot analyses and densitometrically measured. Expression levels of Lyn were measured as the loading control. The relative expression levels were presented as the means±S.E.M. (n=3). The values $^*p<0.05$ are regarded as significant by one-way ANOVA with Dunnett multiple comparison test (*vs.* Day-0).

Mouse Gfi1 and Gfi1b cDNAs were subcloned by reverse transcription polymerase chain reaction (RT-PCR) using the specific primer pairs. They were inserted in pLenti6 vector. Recombinant lentiviruses were prepared using ViraPower Lentiviral Expression System as described previously.[16]

Cell Culture and Lentiviral Expression Connective tissue type cultured mast cells derived from murine bone marrow cells were prepared as described previously.[4] An IL-3-dependent murine mast cell line, MC9, was grown in RPMI-1640 containing 10% heat inactivated fetal bovine serum (FBS), 0.1 mM nonessential amino acid, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 50 μM β-mercaptoethanol and 1 ng/mL of IL-3 in fully humidified 5% CO_2 atmosphere. For lentiviral transduction, MC9 cells were incubated in the presence of the recombinant virus solutions containing 6 μg of polybrene for 24 h at 37°C. The infected cells were then concentrated by selection with 2.5 μg/mL blasticidin S.

Co-culture of MC9 with Swiss 3T3 Cells Swiss 3T3

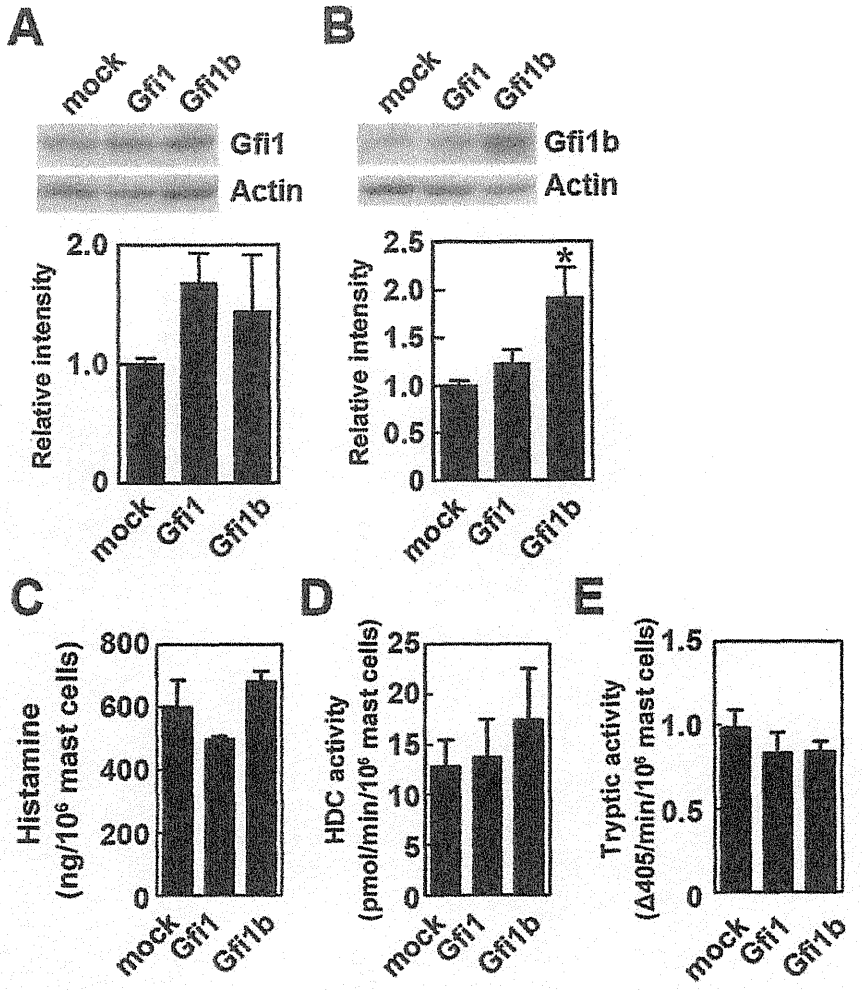

Fig. 2. Effects of Stable Expression of Gfi1 and Gfi1b on Histamine Synthesis and Tryptase Expression in MC9 Cells

Expression of Gfi1 and Gfi1b were enhanced in MC9 cells using the recombinant lentivirus encoding Gfi1 and Gfi1b cDNA (Gfi1, MC9/Gfi1 and Gfi1b, MC9/Gfi1b). (A, B) Expression levels of Gfi1 and Gfi1b were determined by immunoblot analyses and densitometrically measured. Expression levels of β-actin were measured as the loading control. The relative expression levels were presented as the means±S.E.M. (n=3). The values *p<0.05 are regarded as significant by one-way ANOVA with Dunnett multiple comparison test (*vs.* mock). These cells were characterized by measuring histamine content (C), HDC activity (D) and tryptic activity (E). The values were represented as the means±S.E.M. (n=3).

fibroblasts were seeded with 50% confluency in the same medium without IL-3, treated with 3 μg/mL mitomycin C for 3 h, and further incubated for 3 h in the fresh medium without mitomycin C. MC9 cells were seeded on this Swiss 3T3 cells and co-cultured for 24 h or 48 h in the presence of SCF (30 ng/mL).

Measurement of Histamine Content and L-Histidine Decarboxylase (HDC) Activity MC9 cells were lysed in 10 mM potassium phosphate, pH 6.8, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5′-phosphate, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM benzamidine, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL, E-64, 1 μg/mL pepstatin A and 0.1% Triton X-100, and incubated for 15 min on ice. The lysate was centrifuged at 10000×g for 15 min and the resultant supernatant was subjected to measurement of histamine content and HDC activity as described previously.[16]

Immunoblot Analysis MC9 cells were lysed in 20 mM Tris–HCl, pH 7.5, containing 2 mM EGTA, 2 mM EDTA, 1% Triton X-100, 0.5% Na–deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.2 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin, and incubated for 30 min on ice. The lysate was centrifuged at 10000×g for 15 min at 4°C. The resultant supernatant was subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblot analysis was performed as described previously.[16]

Tryptic Activity Assay MC9 cells were lysed in PBS containing 2 M NaCl and 0.5% Triton X-100 for 30 min on ice. The lysate was centrifuged at 10000×g fro 30 min at 4°C. Activity of tryptase in the lysate was measured using S-2288 as described previously.[4]

RESULTS AND DISCUSSION

Induction of Tryptase and Histamine Synthesis in MC9 Cells Co-cultured with Swiss 3T3 Fibroblasts We first confirmed the expression profiles of Gfi1 and Gfi1b in BMMCs during the co-culture period. The expression levels of Gfi1 were transiently increased on Day-4, whereas those of Gfi1b diminished over time, indicating the dynamic changes

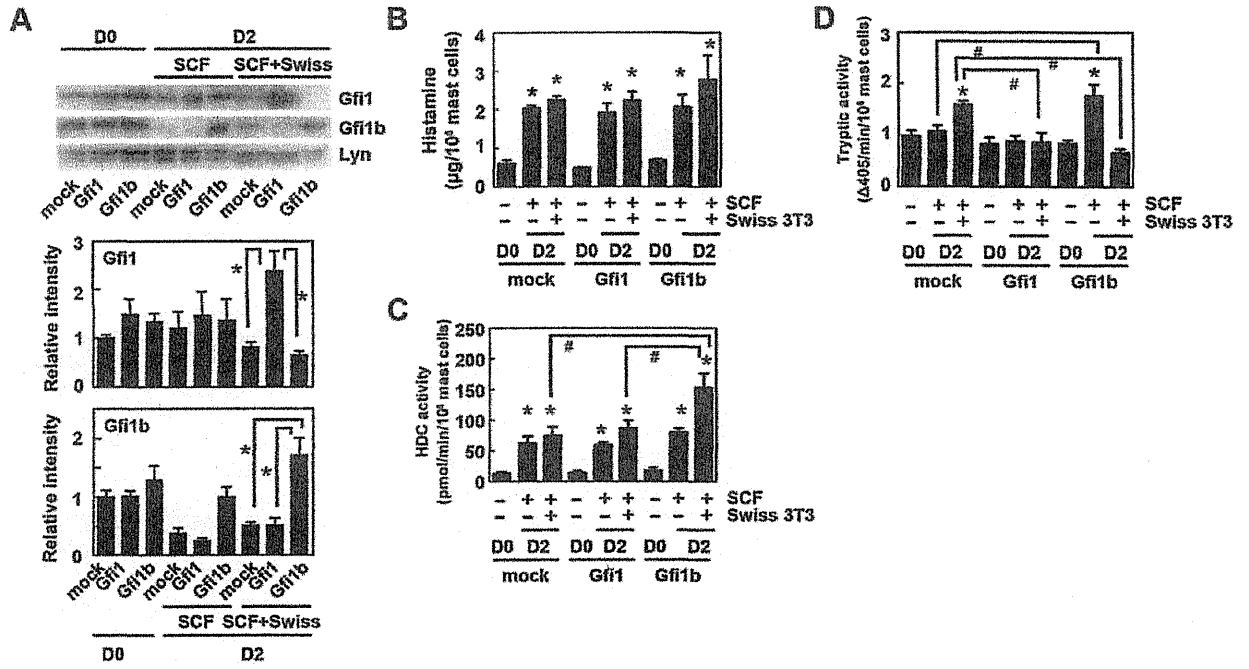

Fig. 3. Effect of Swiss 3T3 Co-culture on CTMC-Like Phenotype in MC9 Cells Transduced with Gfi1 and Gfi1b

MC9 cells without or with lentivirally-transduced Gfi1 and Gfi1b genes (mock, MC9/mock, Gfi1, MC9/Gfi1, and Gfi1b, MC9/Gfi1b) were cultured with or without Swiss 3T3 cells in the presence of SCF (30 g/mL) for 2 d. (A) Expression levels of Gfi1 and Gfi1b in the cells was determined by immunoblot analyses and densitometrically measured. The relative expression levels were presented as the means±S.E.M. (n=3). The values $^{*}p<0.05$ are regarded as significant by one-way ANOVA with Tukey–Kramer multiple comparison test. Expression levels of Lyn were measured as the loading control. These cells were characterized by measuring histamine content (B), HDC activity (C) and tryptic activity (D). The values were represented as the means±S.E.M. (n=3). The values $^{*}p<0.05$ and $^{\#}p<0.05$ are regarded as significant by one-way ANOVA with Tukey–Kramer multiple comparison test.

in expression of Gfi1 and Gfi1b during the co-culture period (Fig. 1A). Maturation of mast cells is usually evaluated by their granule maturation, such as histamine storage and mast cell-specific granule protease expression. Histamine synthesis, which is mediated by L-histidine decarboxylase (HDC), was induced by SCF in the presence or absence of Swiss 3T3 fibroblasts (Figs. 1B, C), whereas tryptic activity was significantly augmented only in the presence of SCF and Swiss 3T3 cells (Fig. 1D). Enzyme activities of chymase and carboxypeptidase A were undetectable in MC9 cells under all conditions investigated (data not shown). We then investigated the expression profiles of Gfi1 and Gfi1b in MC9/mock cells. Since tyrosine kinase, Lyn, is not expressed in Swiss 3T3 cells (data not shown), the amount of Lyn was measured as the loading control. We also confirmed that Gfi1 and Gfi1b were not expressed in Swiss 3T3 cells (data not shown). MC9/mock cells expressed both Gfi1 and Gfi1b under steady state conditions. SCF alone did not affect the expression levels of Gfi1 but down-regulated Gfi1b (Fig. 1E). Co-culture with Swiss 3T3 cells transiently down-regulated Gfi1 and abolished the expression of Gfi1b on Day-2. These results suggest that histamine synthesis should be independent of the expression Gfi1 and induction of tryptic activity may be prevented by Gfi1b.

Effect of Stable Expression of Gfi1 and Gfi1b in MC9 Cells We then investigated the effects of stable expression of Gfi1 and Gfi1b, which was performed using the recombinant lentivirus, on tryptase expression and histamine synthesis. We confirmed that the lentivirally-transduced genes were expressed in MC9 cells by immunoblot analysis (Figs. 2A, B). The amount of Gfi1 was not significantly changed in the cells with the transgenes, whereas that of Gfi1b was increased in the cells expressing the exogenous Gfi1b transgene. It might be unlikely that lentiviral expression of Gfi1 was unsuccessful in this system because the expression levels of Gfi1 were significantly higher in the cells harboring the transgene under the co-culture condition (Fig. 3A). Gfi1 and Gfi1b were found to repress their own transcription and Gfi1b was reported to repress Gfi1 transcription in T cells.[17–19] Exogenously-transduced Gfi1 and Gfi1b proteins may regulate the endogenous expression of Gfi1 and Gfi1b. We then investigated histamine synthesis and tryptase expression in these cells with the transgenes and found no significant changes (Figs. 2C–E), indicating that augmented expression of Gfi1b alone could not affect histamine synthesis and tryptase expression.

Effects of Stable Expression of Gfi1 and Gfi1b in MC9 Cells during the Co-culture Period SCF and co-culture with Swiss 3T3 cells were found to affect the expression levels of Gfi1 and Gfi1b (Fig. 1E), raising the possibility that stable expression of Gfi1 and Gfi1b may alter the phenotypic changes observed during the co-culture period. Forced expression of Gfi1 resulted in enhancement of total Gfi1 expression in MC9 cells in the presence or absence of Swiss 3T3 cells (Fig. 3A). On the other hand, forced expression of Gfi1b augmented total expression of Gfi1b. The profiles of histamine content under three different culture conditions were not affected by the transgenes (Fig. 3B), whereas HDC activity was significantly up-regulated in the cells expressing the Gfi1b transgene in the presence of Swiss 3T3 cells, indicating the positive roles of Gfi1b in gene expression of HDC (Fig. 3C). Relationship between tryptase expression and Gfi1/Gfi1b expression was found to be more complicated. Induction of tryptase expression during the co-culture period was significantly suppressed

in both kinds of MC9 cells expressing the Gfi1 transgene and the Gfi1b transgene (Fig. 3D), indicating that one-sided expression of Gfi1/Gfi1b suppressed the induction of tryptase. In accord with this hypothesis, comparable expression of Gfi1 and Gfi1b, which was observed in MC9 cells expressing the Gfi1b transgene in the presence of SCF alone and in MC9 cells expressing the mock transgene co-cultured with Swiss 3T3 cells, did not prevent the induction of tryptase.

Gfi1 and Gfi1b are involved in regulation of growth and differentiation of various hematopoietic lineages, such as B cells, T cells, neutrophils, dendritic cells, macrophages, and erythrocytes. Here we add mast cells in this list. Gfi1 and Gfi1b have been identified as transcription repressors. For instance, in neutrophils, Gfi1 represses transcription of neutrophil elastase-2 (ELA2), and matrix metalloproteinase-8 (MMP8) by binding to their promoter regions.[11,20,21] Since we previously demonstrated that activated murine neutrophils express HDC,[22] it is possible that Gfi1 controls gene expression of HDC in neutrophils. Gfi1 and Gfi1b were found to share with the same consensus DNA sequence, which consists of 12-base-pairs TAAATCAC(A/T)GCA with a absolute requirement of AATC.[23,24] The possible candidate sites for Gfi1/Gfi1b binding were found in 5′-upstream region of the *Hdc* gene and the *Mcpt6* gene, which encodes a tryptase, mouse mast cell protease-6; −123 (5′-CAAATCAGAAAG-3′) and −861 (5′-GAAATCATATTC-3′) from the transcription initiation site of the *Hdc* gene, and −114 (5′-TAAATCACTATT-3′) and −998 (5′-AAAATCAACTGA-3′) from that of the *Mcpt6* gene. Gfi1 and Gfi1b may bind to these sites and modulate the transcription of the *Hdc* and *Mcpt6* gene.

Gfi1 was found to enhance STAT3-mediated signaling in T cells by directly interacting with PAIS3, which is an inhibitor of STAT3.[25] In mast cells, PIAS3 was reported to interact with a transcription factor MITF and to suppress its functions.[26–28] Since MITF is one of the primary regulators of mast cell differentiation and regulates expression of granule proteases including tryptases,[29] modulation of tryptase expression through Gfi1b may involve MITF in MC9 cells. In summary, we demonstrated that both Gfi1 and Gfi1b might be involved in the process of maturation of murine mast cells.

Acknowledgment This study was supported in part by JSPS KAKENHI (Grant Number 23590077).

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第1特集 3

iPS細胞由来マスト細胞を用いた難治性疾患の新規治療薬開発へ向けて

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Key words　マスト細胞／iPS 細胞／創薬応用／炎症性腸疾患／多発性硬化症

幹細胞と iPS 細胞の違い

幹細胞は、様々な細胞へと分化する能力（多能性）を有しており、適切な条件下で培養することにより、血液細胞、神経細胞、心筋細胞など多種多様な細胞へと分化可能である。したがって、幹細胞は、薬効評価・安全性薬理試験などの創薬スクリーニングや再生医療への応用が期待されている（図1）。

幹細胞のなかでも、とくに人工多能性幹細胞すなわち iPS 細胞は、皮膚などの体細胞に数種の遺伝子を導入することにより樹立されることから、受精卵を用いる ES 細胞（胚性幹細胞）とは異なり、倫理的な問題を回避することができる。また、iPS 細胞は患者自身の細胞から樹立することができるため、移植細胞による拒絶反応の可能性が低いと考えられている。

iPS 細胞の応用においては、神経変性疾患であるパーキンソン病の患者から iPS 細胞を樹立し、目的の細胞に分化させることで、これまで技術的に不可能であっ

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2010年、大阪大学大学院薬学研究科博士後期課程修了。専門は免疫学。ES/iPS細胞から各種血液・免疫細胞への分化誘導法の確立、マスト細胞の分化・成熟化メカニズムの解明に向けて研究中。日本遺伝子治療学会H23年度アンジェスMG賞。研究室リンク

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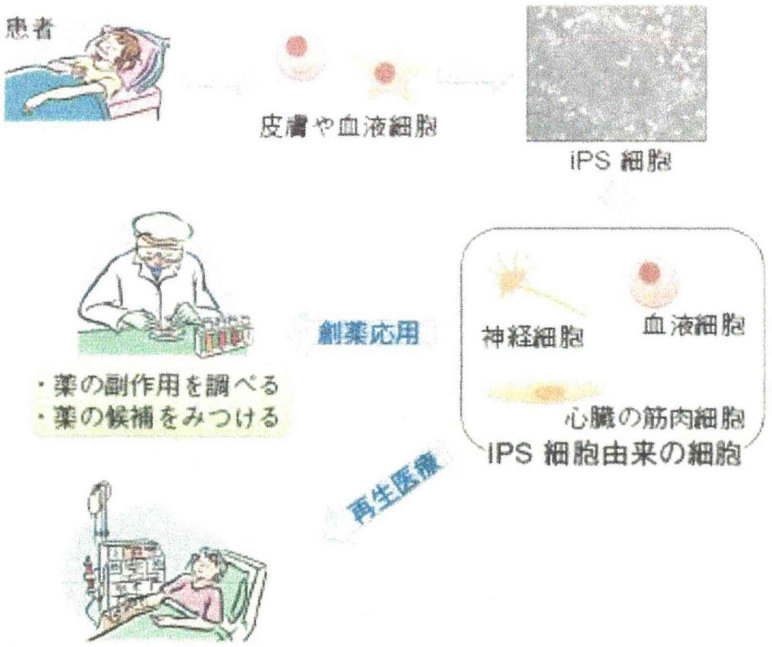

図1　iPS 細胞を用いた臨床応用

た患者の体内組織（パーキンソン病であれば神経細胞）における病態を再現できることが報告されている[1]。

また、アルツハイマー病の患者から樹立した iPS 細胞を用いて、青魚に含まれる不飽和脂肪酸の一種がその発症を抑える可能性があることも報告されている[2]。したがって、発症の原因が未だ不明な疾患のメカニズム解明や新規治療薬の開発に、患者由来 iPS 細胞が有用であることが示されている。他にも、進行性骨化性線維異形成症や原発性免疫不全症候群などの難治性疾患の患者から iPS 細胞が樹立され、現在研究が進められている。

難治性疾患とマスト細胞

炎症性腸疾患は、長期にわたり消化管に原因不明の炎症を呈する難治性疾患であり、近年急速に罹患率が増加している。大腸の粘膜にびらんや潰瘍ができる潰瘍性大腸炎や大腸および小腸の粘膜の慢性の炎症、あるいは潰瘍ができるクローン病などは、炎症性腸疾患の代表的な疾患として知られている。

これらの疾患の病因は未だ明らかとなっておらず、現在では遺伝的因子と環境因子が複雑に絡み合っていると考えられている。

潰瘍性大腸炎やクローン病患者の腸管では、マスト細胞が増加していることから、マスト細胞がこれらの炎症性疾患の発症や増悪に関与していることが、かねてより示唆されていた。

マスト細胞は、蕁麻疹や花粉症といった即時型アレルギーにおいて刺激に応じてヒスタミンをはじめとする多様な炎症性メディエーター（起炎性物質）を細胞外に放出することから、アレルギー応答における主要なエフェクター細胞*1として機能することが知られている。しかし、即時型アレルギー以外におけるマスト細胞の生体内での役割については不明であり、現在少しずつ解明されている。近年、遺伝的にマスト細胞を欠損したマウス（C57BL/$6^{W\text{-}sh/W\text{-}sh}$ マウス）を用いて実験的に炎症性腸疾患を誘導したところ、野生型マウスと比較して炎症が軽減されることが報告されたことから、マスト細胞が炎症性腸疾患の病態の悪化に関与していることが示されている[3]。

多発性硬化症は、脳や脊髄などの中枢神経系が炎症を起こすことにより、神経が障害される難治性疾患である。手足の麻痺や視力の低下などの重篤な症状が現れ、その症状は悪化と好転を繰り返すことを特徴とする。多発性硬化症の発症原因に関しては明らかになっていないが、近年の研究によりアレルギーや炎症性腸疾患だけでなく、多発性硬化症の発症や増悪にもマスト細胞が関与していることが示唆されている[4]。実際、多発性硬化症の患者の脳に発現する遺伝子プロファイルを解析した研究では、ヒスタミン受容体、マスト細胞が有するプロテアーゼ、その他の炎症性メディエーターなど、マスト細胞由来の遺伝子やアレルギー炎症で発現が上昇する遺伝子の発現量が増加していることも報告されている。

ES/iPS 細胞由来マスト細胞

前述したように、マスト細胞は種々の疾患の発症や増悪に関与していることが報告されていることから、これらの疾患においてマスト細胞を標的とした新規治療薬の開発が期待される。しかしながら、マスト細胞は、生体では皮膚等の組織に浸潤して存在しており、その数も少ないため生体から取り出して培養することは容易ではない。そこで我々は、iPS 細胞から効率良くマスト細胞を分化誘導可能な培養系の確立を試みた[5]。iPS 細胞からマスト細胞を得るには、様々な血液細胞への分化能を有する血液前駆細胞、マスト細胞前駆細胞を分化誘導する必要がある。まず、マウス ES 細胞あるいは iPS 細胞から血液細胞を得る際に汎用され

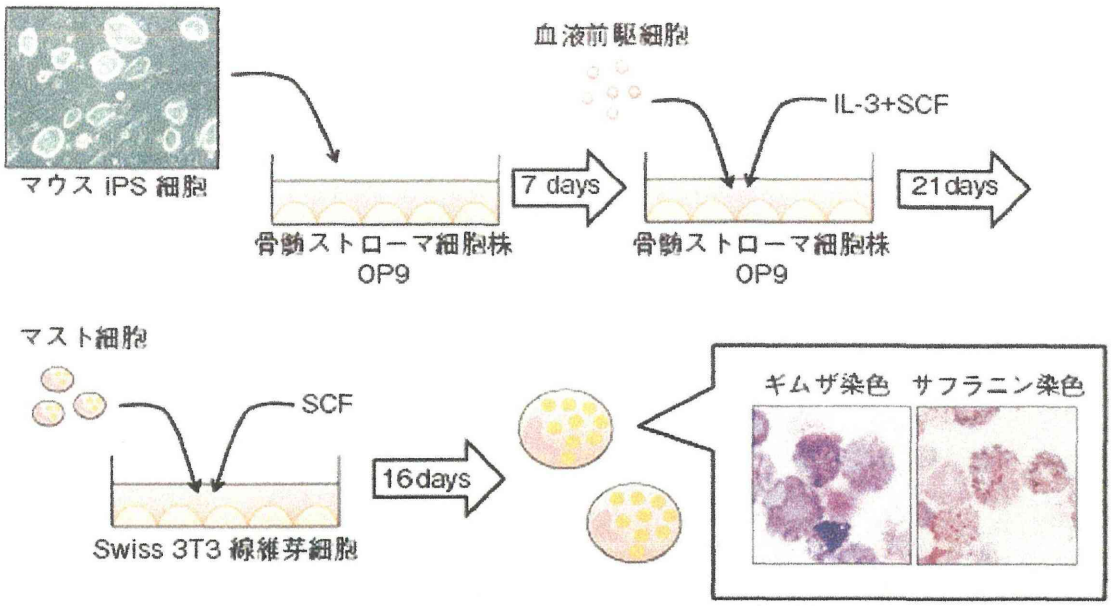

図 2　iPS 細胞からマスト細胞への分化誘導法

ている骨髄ストローマ細胞株である OP9 細胞と共培養することにより血液前駆細胞を誘導した。その後、マスト細胞への分化に有用とされているインターロイキン 3（IL-3）や幹細胞増殖因子（stem cell factor: SCF）存在下で、血液前駆細胞を OP9 細胞と 21 日間共培養することでマスト細胞を得た（図 2）。得られた iPS 細胞由来マスト細胞は、マスト細胞研究で汎用されている IL-3 依存性骨髄由来マスト細胞と比較し、マスト細胞特異的酵素活性やマスト細胞の重要な機能である脱顆粒（細胞内に蓄積している顆粒を刺激に応じて細胞外に放出する）応答能の亢進が観察されたことから、より成熟度の高いマスト細胞であることが明らかとなった。さらに、得られた iPS 細胞由来マスト細胞を SCF 存在下で Swiss 3T3 線維芽細胞と共培養することで、生体に存在するマスト細胞と同様に、ヒスタミンなどの炎症性メディエーターを豊富に含む顆粒が多く存在するマスト細胞を分化誘導可能であることが示された（図 3）。

上述したように、炎症性腸疾患や多発性硬化症などの発症や悪化にマスト細胞が関与していることから、ヒト iPS 細胞由来マスト細胞を用いることで、これらの疾患に対してこれまでの治療薬とは作用点が異なる新規治療薬の開発が可能となる。

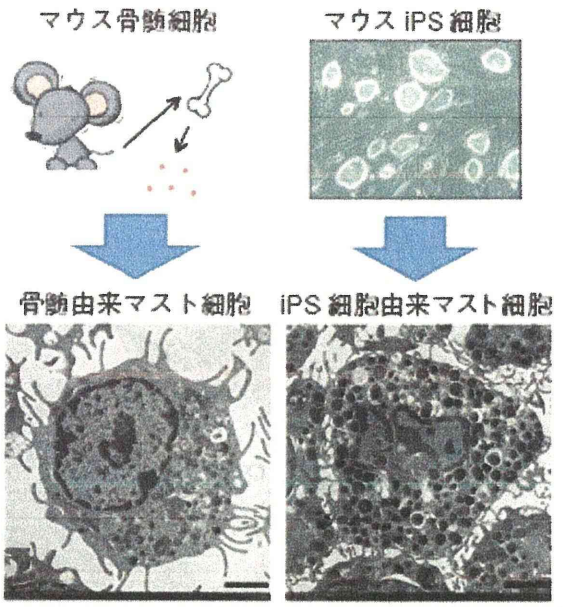

図3　iPS 細胞由来マスト細胞の表現型

マスト細胞のように、生体から取り出し培養することが困難な細胞は、iPS 細胞を用いた創薬応用に適した標的細胞であると考えられる。今後、ヒト iPS 細胞由来マスト細胞を効率良く分化誘導する方法が確立され、それを用いた難治性疾患の新しい治療薬が開発されることを期待している。

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【用語解説】

＊1　エフェクター細胞：T 細胞のうち、細胞性抗体が反応に関係する遅延型アレルギー（Ⅳ型アレルギー）に関与するサイトカインを産生する細胞。