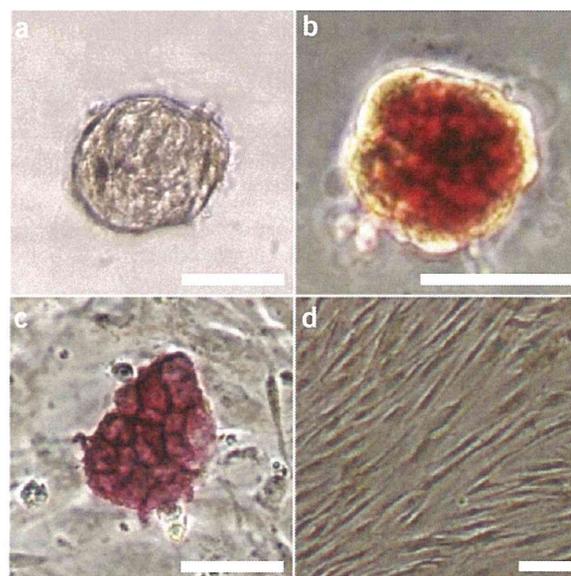


Figure 5 | Characterization of M-clusters. (a) Phase-contrast image of an M-cluster. (b–d) The result of the ALP reaction; the M-cluster (b) and mouse ES cells (c) are positive for the ALP reaction, whereas NHDFs (d) are negative. Scale bars, 50 μm (a–c), 100 μm (d).



Muse cells can be purified from the mesenchymal cell populations such as adult human BMSCs, dermal fibroblasts and the mononuclear cell population of fresh bone marrow by FACS using the antibody against the specific cell surface marker SSEA-3 (Fig. 4). The percentage of SSEA-3⁺ cells among these cell populations is dependent on the cell sources: it is <1% in adult human cultured BMSCs, 2–3% in adult human cultured fibroblasts and 0.003–0.004% in mononuclear cells directly isolated from fresh bone marrow. Muse cells have telomerase activity as low as that of naive fibroblasts or BMSCs (Supplementary Fig. 1), suggesting low tumorigenicity of Muse cells. After SSEA-3⁺ cells are isolated, they can be cultured in a single-cell suspension culture or an MC culture. About half (40–60%) of the Muse cells form M-clusters, whose diameters are >25 μm and which resemble embryoid bodies generated from human ES cells, have ALP activity similar to that of other pluripotent cells (Fig. 5) and contain the cells positive for pluripotency markers such as Nanog, Oct3/4 and Sox2 in addition to SSEA-3 (as shown by immunocytochemical analysis and RT-PCR (Fig. 6)). The growth curve of Muse cells in M-cluster formation shows the limitation of proliferative activity of Muse cells in a single-cell suspension culture (Supplementary Fig. 2). The proportion of M-cluster-forming activity is dependent on the viability of cells and is usually 45–65% in our laboratory. In contrast, SSEA-3⁻ and/or CD105⁻ cells did not form any clusters after single-cell suspension culture or MC culture. After expansion of M-clusters in adherent culture, second-generation M-cluster can be formed from the M-cluster-derived adherent culture-expanded cells, and the proportion of second-generation M-cluster formation is $48.0 \pm 5.8\%$ (fibroblasts) (mean \pm s.d.) or $40.3 \pm 9.1\%$ (BMSCs). Alternatively, Muse cells can be isolated by FACS sorting with anti-SSEA-3 antibody, and the percentage of SSEA-3⁺ cells is $45.0 \pm 3.2\%$ (fibroblasts). This SSEA-3⁺ percentage is similar to the proportion of M-cluster-forming cells after adherent culture presented above, suggesting that SSEA-3⁺ Muse cells have M-cluster-forming activity.

Differentiation of Muse cells can be confirmed by two methods: a spontaneous differentiation assay in which they are cultured on the gelatin-coated coverslip or induced differentiation of Muse cells into mesodermal-, endodermal- and ectodermal-lineage cells. After culturing on a gelatin-coated coverslip with 10% (vol/vol) FBS in α -MEM, Muse cells spontaneously differentiate into cells representative of all three germ layers, including cells positive for neurofilament (marker for neuronal cells, ectodermal lineage), SMA (smooth muscle, mesodermal lineage), α -fetoprotein (hepatocyte, endodermal lineage), cytokeratin-7 (biliary duct, endodermal lineage) and desmin (muscle, mesodermal lineage) (Fig. 7a–d). RT-PCR demonstrates the expression

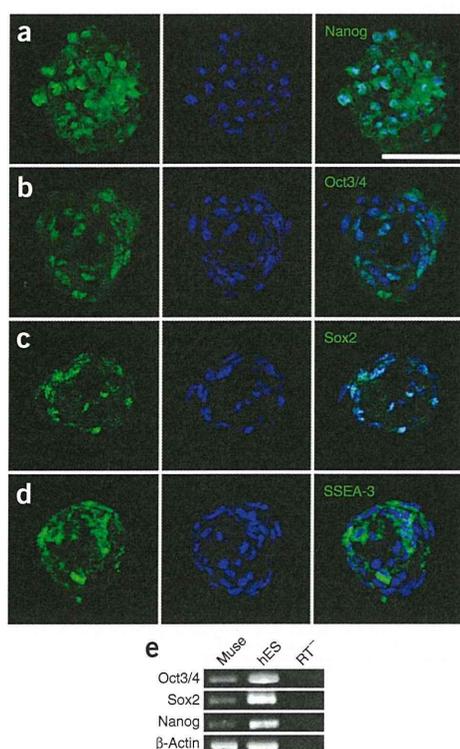


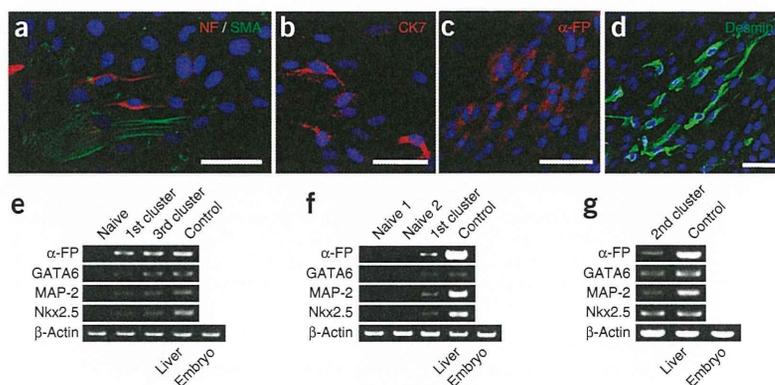
Figure 6 | Expression of pluripotency markers in M-clusters. (a–d) Immunocytochemical analysis showing cells in an M-cluster expressing pluripotency markers (green) such as Nanog (a), Oct3/4 (b), Sox2 (c) and SSEA-3 (d); nuclei are also visualized (blue). (e) mRNA expression of pluripotency markers. Human ES cells are used as a positive control. Scale bar, 50 μm . Samples treated without a reverse-transcription reaction (RT-) are used as a negative control. Panels a, b, e are modified from Kuroda *et al.*³⁹, Wakao *et al.*⁴⁰ and Kuroda *et al.*³⁹, respectively.

PROTOCOL

Figure 7 | Spontaneous differentiation of Muse cells on a gelatin-coated coverslip.

(a–d) Immunocytochemistry against markers for specific cell types; M-cluster-derived cells cultured on gelatin-coated coverslips are positive for neurofilament NF and SMA (a), cytokeratin-7 (CK7) (b), α -fetoprotein (α -FP) (c) and desmin (d).

(e–g) Expression of mRNA for cell lineage markers. M-cluster-derived cells cultured on the gelatin-coated coverslip express mRNA for α -FP, GATA6, MAP-2 and Nkx2.5 (e, cells from M-clusters derived from adult human dermal fibroblasts; (f,g) cells from M-clusters derived from a mononucleated cell of human fresh bone marrow). Human fetal liver (liver) was used as a positive control for α -FP, and whole human embryo (embryo) was used as a positive control for GATA6, MAP-2 and Nkx2.5. Scale bars, 50 μ m. Panels a,g are modified from Wakao *et al.*⁴⁰ and Kuroda *et al.*³⁹, respectively.



of α -fetoprotein, GATA6 (endodermal lineage), MAP-2 (ectodermal lineage) and Nkx2.5 (mesodermal lineage) (Fig. 7e–g). Muse cells without induction of differentiation do not express any of the differentiation markers mentioned above, as shown by immunocytochemical analysis and RT-PCR (Supplementary Fig. 3).

The self-renewal property of Muse cells can be confirmed by cycle culture consisting of suspension culture–adherent culture–suspension culture. M-cluster in the third generation created by this cycle-culture method also has the same differentiation ability as that observed in the first generation (Fig. 7e), indicating the self-renewal property of Muse cells. Muse cells can differentiate into mesodermal-, endodermal- and ectodermal-lineage cells under the directed method. For example, Muse cells give rise to osteocalcin-positive osteocytes (Fig. 8) and adipocytes (mesodermal lineage) that are detected as cells containing lipid droplets inside the cytoplasm (Fig. 8b), and which are positive for oil red staining (Fig. 8c). These cells are representative of the mesodermal lineage. Hepatocytes (endodermal lineage) that express α -fetoprotein and human albumin are able to be produced from Muse cells after culturing with 10% (vol/vol) serum-containing medium supplemented with several hormones and trophic factors, including dexamethasone, HGF and FGF-4 (Fig. 8d,i). In the suspension culture system containing bFGF and EGF in a serum-free medium, Muse cells can also give rise to neural progenitor cells, which are positive for nestin (Fig. 8e), musashi-1 (Fig. 8f) and NeuroD (Fig. 8g) (ectodermal lineage). These neural progenitor cells differentiate into MAP-2⁺ neurons upon treatment with bFGF and BDNF in adherent culture (Fig. 8h). These procedures confirm Muse cells' capacity for differentiation and self-renewal.

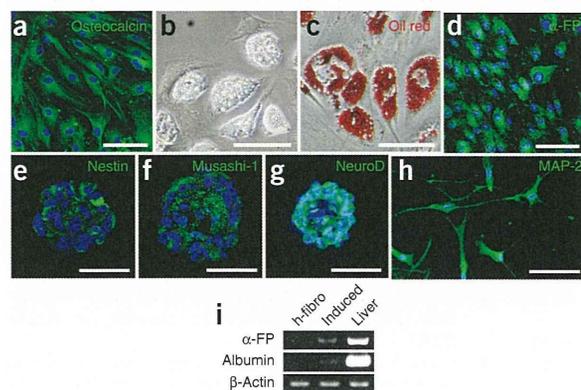


Figure 8 | Induced differentiation of Muse cells into mesodermal-, endodermal- and ectodermal-lineage cells. (a–h) Immunocytochemical analysis of differentiated cells. (a) After osteocyte induction, the cells positive for osteocalcin are detected. Adipocyte induction generates cells with lipid droplets (b) that are positive for oil red staining (c). (d) Hepatocyte induction generates cells positive for α -fetoprotein (α -FP). (e–g) After neural induction, the cells form spheres resembling neurospheres positive for the neural progenitor markers (green) Nestin (e), Musashi-1 (f) and NeuroD (g). (h) This neurosphere-like sphere gives rise to MAP-2⁺ neurons after treatment with trophic factors. (i) mRNA expression in differentiated hepatocytes. M-cluster-derived cells (induced) can differentiate into cells expressing α -FP and human albumin mRNA, whereas naive adult human fibroblasts (h-fibro) do not express both mRNAs. Human fetal liver (liver) was used as a positive control. Scale bars, 50 μ m (a,d–h), 20 μ m (b,c).

Note: Supplementary information is available in the online version of the paper.

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experiments and all the authors prepared the figures. M.K. and M.D. contributed to the description of the protocols.

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ヒト生体に内在する新たな多能性幹細胞 Muse細胞： 細胞治療，予後の診断，創薬，病態解析への展開の可能性

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1. はじめに

間葉系幹細胞は骨髄，脂肪，真皮，臍帯などに存在する組織幹細胞であり，腫瘍化の危険性が低く安全性が高いこと，入手しやすい組織から得られることなどから，臨床試験が各国で精力的に進められている。栄養因子やサイトカインの産生による組織保護効果や抗炎症作用，アポトーシス調節作用をもたらすので様々な疾患を対象に移植が検討されており，また免疫抑制作用も持つことから，移植片対宿主病に対する臨床応用も進められている。

しかし，間葉系幹細胞にはこれらの作用の他に重要な作用がある。それは，胚葉を超えた幅広い分化と生体内での組織修復作用である。これらの作用は長らく科学的に議論されてきたが，間葉系幹細胞が様々な細胞種の混合から成り立っており，そのような幹細胞の本態が明らかにされてこなかったことや，生体内での組織修復効果の率が極めて低いなどの理由から実態がよくわかっていなかった。

我々の研究室ではヒトの間葉系組織や間葉系の培養細胞において，多能性を有するが腫瘍性を持たない新たなタイプの体性幹細胞 Multilineage-differentiating stress enduring (Muse) 細胞を見出した^{1),2)}。この細胞は，成人の生体内に存在する多能性幹細胞であり，これまで間葉系幹細胞で見られてきた幅広い分化能や組織修復を説明すると考えられる。

2. 生体内多能性幹細胞としてのMuse細胞

Muse細胞は間葉系幹細胞の一員であると同時に，多能性幹細胞としての振る舞いを見せる。このような特性はマーカーの発現にも見られ，間葉系マーカーCD29，CD105，CD90と同時に，未分化ヒトES細胞のマーカーstage specific embryonic antigen-3 (SSEA-3) のダブル陽性細胞，すなわち間葉系と多能性幹細胞の両方のマーカーを発現する細胞として同定される。

Muse細胞は1細胞レベルでの浮遊培養を行うと増殖を開始し，ヒトES細胞由来の胚葉体と酷似した細胞塊clusterを形成する¹⁾。このclusterはアルカリフォスファターゼ反応に陽性を示し，Nanog，Sox2，Oct3/4などの多能性マーカーを発現する。さらに，この1細胞から形成された細胞塊をゲラチン上で培養するとsmooth muscle actin (平滑筋)，desmin (骨格筋)，alpha-fetoprotein (肝細胞)，cytokeratin-7 (胆道系細胞)，neurofilament (神経) など三胚葉性の細胞へ自発的に分化する¹⁾。このような自発的な三胚葉性の細胞への分化率は高くはなく，内胚葉性や外胚葉性の細胞が全体の3～4%，中胚葉性の細胞が10～15%であるが，一方，Muse細胞に特定の誘導をかけると90%以上の高い効率で神経，肝細胞，脂肪細胞，骨細胞などの目的とする細胞に分化させることが可能である。従ってMuse細胞からは効率の良いdirect reprogrammingが可能である。

Muse細胞は成人ヒトの皮膚，骨髄などの間葉系組織から採取可能である。生体の間葉系組織の場合，例えば骨髄液では，3,000個の骨髄単核球細胞のうち1細胞の割合で存在する^{1),2)}。生体内でのMuse細胞の局在を組織学的に検討すると，真皮，脂肪組織や他の様々な臓器では結合組織内に孤立性・散在性に存在し，特定の組織構造との関連は

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見られなかった²⁾。また、市販の間葉系の培養細胞では、多少の増減はあるものの大体1%ないし数%程度の割合で含まれている。

3. Muse細胞は多能性であるが腫瘍性がない

腫瘍性増殖能に関わる因子の発現量を調べると embryonic stem (ES) 細胞, induced pluripotent stem (iPS) 細胞は非常に高値を示すが, Muse細胞では体細胞とほぼ同じレベルで低い²⁾。さらにヒト細胞への免疫拒絶を示さない免疫欠損マウス (SCID マウス) の精巣への移植では, ヒト ES細胞や iPS細胞は8~12週で奇形腫を形成するのに対し, ヒト Muse細胞では半年を経過しても奇形腫の形成は全く見られなかった¹⁾。間葉系幹細胞は腫瘍化の危険が低く,すでにヒトに移植されている細胞であり, Muse細胞はその間葉系幹細胞の一部であることと併せて考えると, もともと腫瘍化の危険は低いと思われる。

4. Muse細胞は生体に投与されると組織修復機能をもたらす

ヒト細胞を拒絶しない免疫欠損マウスを用いて劇症肝炎, 筋変性, 脊髄損傷, 皮膚損傷などの様々なモデルを作製し, GFPで標識したヒト Muse細胞を尾静脈から投与すると (ただし皮膚損傷の場合は局所注入を行った), 移植4週後で Muse細胞は傷害部位に生着し, 肝細胞, 筋細胞, 神経細胞, 角化細胞にそれぞれ分化することが確認されている¹⁾。

SCID マウスの腹腔に四塩化炭素を投与して作製した劇症肝炎モデルでは, 尾静脈から投与した GFP陽性のヒト Muse細胞が肝臓の傷害部位に経血管的に生着しており, さらに, 生着した細胞の約90%近くがヒトアルブミンやヒトアンチトリプシンをマウス肝臓内発現することが確認された。マウスの末梢血を採取し Western blot で解析するとヒトアルブミンが検出されたことから, 移植されたヒトの Muse細胞がマウスの肝臓内に生着し, ヒト肝細胞として分化し, さらにヒトのアルブミンを血中に放出することのできる機能的細胞になっていたことを示唆する¹⁾。

同様に, 筋変性モデルに生着したヒト Muse細胞はヒトのジストロフィンを, 損傷脊髄では neurofilament を, 損傷皮膚では cytokeratin 14 を発現していた¹⁾。このように, 投与されたヒト Muse細胞は生体内で損傷部位を認識して生着し, 機能的な細胞に分化すること, さらに三胚葉性の細胞に生体内でそれぞれ分化することが示された。

一方 Muse細胞を除いたヒト間葉系幹細胞, すなわち非 Muse細胞群を同様の方法で生体に投与しても, Muse細胞

で見られたような損傷組織への生着や各組織の細胞への分化は観察されない¹⁾。従って, Muse細胞は損傷部位を認識し, 組織を構成する細胞となりうる組織修復機能を持つが, Muse細胞以外の間葉系幹細胞にはこのような機能が備わっていない, すなわち間葉系幹細胞移植においてこれまで見られてきた組織再生, 組織修復は Muse細胞が担っていると考えられる。

Muse細胞の特性をまとめると,

- ・ 遺伝子導入などの人工的な操作なしに, ヒト生体から直接得られる多能性幹細胞。三胚葉性の多様な細胞に分化できる。
- ・ 入手しやすい皮膚, 骨髄, 脂肪や市販の線維芽細胞などから採取可能である。
- ・ 腫瘍化の危険性が低い。
- ・ 骨髄移植の0.03%, 間葉系幹細胞移植の数%に相当。すでに移植されている細胞の一部であり, 安全性の見通しが高い。
- ・ 培養では線維芽細胞と同程度の増殖力を持ち, 脂肪や骨髄から細胞数確保が可能。
- ・ 生体内にそのまま投与すると組織修復をもたらす。などの特徴に集約される。

5. 再生医療における Muse細胞の可能性と戦略

間葉系幹細胞は組織保護や修復に効果があるとされ, 臨床応用も進められているが, 特に再生効果・組織修復作用に関しては明快な科学的根拠が提示されてこなかった。そのような中で, 三胚葉性の細胞への幅広い分化能力を有する多能性の Muse細胞が間葉系幹細胞の中から同定され, さらにはこの細胞が再生効果, 組織修復効果を担っているということが明らかになったことには再生医学において意義がある。これまで雑駁な間葉系幹細胞をそのまま生体に投与する形で臨床試験などが行われているが, その中に含まれる Muse細胞を精製する, あるいは比率を上げることによって組織修復効率の向上や有効性の高い再生医療への応用が期待される。ただし, Muse細胞以外の間葉系細胞が必要であるのか不要なのか, この点は慎重な判断が必要と思われる。疾患によっては急性の炎症, 組織破壊など複合的な要因が絡み合っている場合がある。このような「場」に Muse細胞だけを投与するよりも, 抗炎症, 抗アポトーシス作用を持ち組織保護をもたらす非 Muse細胞が一定程度存在するほうが, Muse細胞自身の生着, 生存が上がり, 結果として有効な組織再生につながる可能性がある。Muse細胞をどのように有効活用するかは今後の大きな課題である。

本稿の著者には規定されたCOIはない。

文 献

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