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医療技術実用化総合研究事業

ヒト生体由来多能性幹細胞（Muse細胞）の再生医療への応用に向けた安全性・有効性の検証

平成２５年度　総括研究報告書

研究代表者　出沢　真理

平成２６（２０１４）年　３月

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目　　次

Ⅰ. 総括研究報告書

ヒト生体由来多能性幹細胞（Muse細胞）の再生医療への応用に向けた安全性・有効性の検証 ---------------------- 1
出沢　真理

Ⅱ. 研究成果の刊行に関する一覧表 ---------------------- 7

Ⅲ. 研究成果の刊行物・別刷 ---------------------- 9

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総括研究報告書

ヒト生体由来多能性幹細胞（Muse細胞）の再生医療への応用に向けた安全性・有効性の検証

研究代表者　出沢真理　東北大学大学院医学系研究科・教授

研究要旨　Muse 細胞は成人ヒトの骨髄、皮膚などの間葉系組織から多能性幹細胞マーカーと間葉系マーカーの二重陽性細胞として採取可能な生体由来の多能性幹細胞であり、腫瘍性を持たない。3 胚葉性のあらゆる細胞に分化する能力を持ち、そのまま生体内に投与すると損傷部位に生着し、組織に応じて機能的細胞に分化して様々な臓器の再生をもたらす。Muse 細胞を迅速に臨床試験を実現するために、誘導しないそのままの Muse 細胞懸濁液を疾患部位に投与する「医薬品」として開発することを本研究の目的とし、ターゲット臓器を肝臓に絞り非臨床有効性試験、細胞調製の最適化、Muse 細胞製剤の規格の設定、安全性検証等を行った。本年度は急性肝障害モデルを中心に研究を行ったところ、Muse 細胞は肝組織への生着、分化による組織修復を担うが、Muse 以外の非 Muse 細胞にはこのような能力がないことが分かった。Muse 細胞の核型検査を行ったところ、変異等異常は無いことが分かった。Muse 細胞の実用化を推進することにより、幅広い疾患を対象に安定的な効果をもたらす細胞治療が世界に先駆けて実現されると期待される。

研究分担者
浅田隆太
　京都大学医学部附属病院
　臨床研究総合センター・特定助教
清水忍
　名古屋大学医学部附属病院
　先端医療・臨床研究支援センター
　病院講師
藤澤浩一
　山口大学医学部
　修復医学教育研究センター・助教
吉田正順
　(株)Clio 社・代表取締役社長

A．研究目的

間葉系幹細胞は 3 胚葉性にまたがる多様な細胞に分化するので ES 細胞のような多能性幹細胞が内在すると推察されていた。今回その説明となる Muse 細胞が発見された。Muse 細胞は多能性幹細胞としての特性を備えているが腫瘍性が無いところが最大の利点である。

誘導せずに、そのまま生体内に投与すると損傷部位に生着し、機能的細胞に分化して様々な組織再生をもたらす（PNAS, 2010）。また特定の誘導をかけると神経、肝細胞、骨細胞などに 90%以上の高率で誘導も可能である。従っていくつかの利用方法が想定できるが、迅速に臨床試験を実現するためには、誘導しないそのままの Muse 細胞懸濁液を疾患部位に投与する「医薬品」として開発が最も現実的であると考えられる。Muse 細胞はすでにヒトに移植されている骨髄や間葉系細胞に含まれているとはいえ、精製した細胞を投与する際の安全性・有効性評価は必須である。本研究では PMDA の審査官として薬事承認審査等の経験を有する浅田隆太と民間企業（株）Clio 社が研究分担者として参画し、実用化を目指した検証を行う。

ターゲット臓器として肝臓を選択する。血中アルブミンやビリルビンなどの機能改善や有効性を計るための客観的指標があること、また Muse 細胞が含有されている骨髄細胞を用いた治療（自己骨髄細胞投与療法 ABMi）が研究分担者藤澤浩一の所属する山口大学で肝硬変患者に実施されており、有効性・安全性を計るためのパラメーター設定が明確であるためである。

本プロジェクトでは、①有効性検証の指標設定、②非臨床有効性試験（急性と慢性の肝疾患モデルを用いた有効性検証、用量探索、投与速度の

最適化）、③細胞調製の最適化、④Muse 細胞製剤の規格の設定、⑤非臨床安全性評価を実施する。①、③、⑤の一部（in vitro の安全性評価）はほぼ終了しており、H24～H25 に②、③、④を実施し、その後、⑤（in vivo の安全性評価を中心）を行う。また、薬事戦略相談を適宜利用して、安全性が高く再生効果を有する Muse 細胞を出来る限り早く臨床試験に移行できるように進める。

B．研究方法

【急性・慢性肝障害モデルを用いたヒト Muse 細胞の有効性評価、用量探索試験、投与速度の最適化】

H24 年：モデル作製の条件検討を行った。ヒト細胞を拒絶しない免疫不全動物の SCID マウスを使用する。急性肝障害モデルは腹腔内に四塩化炭素 CCl4 を 1.0mg/Kg の量で投与し、モデルとして最適の作成量を決定した。慢性モデルは週に 2 回、1.0mg/Kg の分量を 4 週間にわたって投与し作成することとした。

ヒト Muse 細胞は骨髄（あるいは脂肪、皮膚）などから FACS で SSEA-3/CD105 ダブル陽性細胞として採取する。FACS によって Muse 細胞を除去した残りの間葉系細胞群を非 Muse 細胞とし、尾静脈投与するヒト Muse 細胞の細胞数は 2 万とし、急性・慢性モデルに投与する。速度投与の最適化を検討する。血清アルブミン量、総蛋白量、ビリルビン値の他、体重、一般血液、血液生化学、全身状態、組織学的検討による線維化領域の算出、などによって有効性評価を 7 週で行う。

【細胞調製の最適化，Muse 細胞製剤の規格の設定】

本研究では急性・慢性の肝障害モデルに上記で凍結保存した Muse 細胞の移植を行い、FACS で採り立ての細胞との比較検討を行い検証する。

【非臨床安全性評価】

Clio 社が Muse 細胞製剤の製造工程の目途を立てる。

【薬事戦略相談】

H26 年：非臨床有効性試験が終了した段階で、非臨床安全性試験（毒性試験・安全性薬理試験等）の計画について、薬事戦略相談を行う。

・非臨床安全性試験（毒性試験・安全性薬理試験等）が終了し、治験デザインの骨子が固まった段階で、治験デザイン、Muse 細胞製剤の品質等について相談を行う。

（倫理面への配慮）

東北大学と山口大学の遺伝子組換え実験安全専門委員会と動物実験専門委員会の指針に従って研究計画書を提出し、機関承認を得た後に実験を実施しており、承認を得た計画のみを実行する。

ヒト間葉系細胞はスイス Lonza 社やアメリカの ATCC などから購入した細胞を用いる。ヒト各種組織、臓器などは同じく購入によって入手したものを実験に用いる。

遺伝子導入実験は「東北大学　遺伝子組み換え実験計画承認」を得ている（研研 76－20－35 号）。また動物実験委員会の承認に関しては 2011 医動－282、2011 医動－283、2011 医動－284、2011 医動－285 にて承認を得た上で実験を行っている。

C．研究結果

投与速度の最適化をヌードマウスとヒト線維芽細胞を用いておこなった。呼吸不全・肺塞栓などの有害事象を検討したところ 9 匹のヌードマウスすべてにおいて、2 万細胞を 30 秒で尾静脈から投与しても morbidity と mortality はみられなかったのでこの速度投与を採用した。

SCID マウスでの急性・慢性肝障害モデルを作成し、ヒト骨髄由来 Muse 細胞の有効性を検討した。急性モデルではヒト骨髄由来 Muse、非 Muse それぞれ 2 万細胞を四塩化炭素投与 2 日目に SCID マウス尻静脈より投与した。いずれの群も総ビリルビンの上昇がマイルドに抑えられた他は、GOT、GPT、総蛋白、アルブミンは一過性に増悪し、14 日前後には正常に戻ってしまい統計的な有意差は見られなかった。しかし組織学的検討では顕著な差があり、移植 30 日後では非 Muse 細胞はほとんど肝臓組織内に残っておらず検出されなかった一方、Muse 細胞は組織内に生着し、Heppar 1, albumin, anti-trypsin など肝細胞の機能的なマーカー陽性を示していた。

慢性モデル実験も行い、Muse 細胞投与群では肝臓の線維化の有意な抑制効果を認め、ヒト Muse 細胞の肝臓内での生着、分化が急性モデル同様に確認されている。また線維化抑性については非 Muse 細胞とは優位差をもって有効性を示すデーターが得られている。

Muse 細胞の安全面の検証として、健康成人に由来する骨髄由来の Muse 細胞の核型検査を実施し染色体数の異常が見られないことが確認された。検証は一般財団法人食品薬品安全センター秦野研究所で実施された。臨床応用を目指した Muse 細胞の分離方法の確立に関してはトリプシンと物理的ストレスを組み合わせた方法で約 30%、AutoMACS では 70～80%のの精製率で得られることが確認できた。

これらの結果を持って H25 年 10 月 24 日に PMDA と薬事戦略相談を行った。

Clio 社はこれらの成果をもとに検討し、現在 Muse 細胞製剤作成のための MACS を用いた製造工程を検討している。

進捗状況としてはほぼ当初の予定通りに進んでいると考えている。

D. 考察

期待される成果は、安全性が高い様々な疾患を対象とした再生医療の実現である。

間葉系幹細胞は肝硬変、心筋梗塞、脊髄損傷、脳梗塞、変形性関節症などの多岐にわたる疾患において、ドイツ、イギリス、アメリカを含め、世界中で臨床試験が展開され一定の効果を挙げている。もたらす効果として、間葉系幹細胞の産生するサイトカインによる保護効果の他に、投与したごく一部の細胞が損傷を受けた組織に生着して分化し、失われた細胞を「置換」する再生効果が報告されている。ただサイトカイン効果は一過性であり、持続的な機能回復は欠落した細胞の置換による再生効果が担っている。このような細胞の探索が世界中で進められていたが、今回Muse細胞が再生効果を担うことが明らかになった。

Muse細胞では劇症肝炎、筋変性、脊髄損傷、皮膚損傷での組織再生が確認されており、実用化を推進することにより、幅広い疾患を対象に安定的な効果をもたらす細胞治療が世界に先駆けて実現されると期待される。開発にしのぎを削っている細胞治療において、日本の先駆性・牽引性がもたらされ、社会的な意義と国民の健康維持への貢献は大きい。また医薬品としてのMuse細胞製剤が臨床応用されることにより新たな医療創生となり、経済効果と新規雇用創出にもつながり厚生労働行政に貢献すると期待される。

E. 結論

急性および慢性肝障害モデルを用いて、血管から投与された Muse 細胞が傷害組織に遊走、生着し、さらに機能的な肝細胞に自発的に分化することが示唆された。一方、間葉系幹細胞の大半を構成する非 Muse 細胞にはこのような効果が無いことが分かった。Muse 細胞はこのように間葉系幹細胞の中から精製し投与することにより有効な再生医療が期待されるが、Muse 細胞製剤を作るに当たり、日本だけでなく欧米でも承認の取れていない FACS で精製するのではなく、すでに FDA でも承認を受けた MACS で進めるのが現状では妥当ではないか、との結論を得た。これらのデーターや現状を踏まえ、さらに PMDA との面談を進めて行く。

F. 危険情報

無し

G. 研究発表

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H. 知的財産権の出願・登録状況

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Review Article

Muse cells, newly found non-tumorigenic pluripotent stem cells, reside in human mesenchymal tissues

Shohei Wakao,[1] Hideo Akashi,[1] Yoshihiro Kushida[2] and Mari Dezawa[1,2]

Departments of [1]Stem Cell Biology and Histology, and [2]Anatomy and Anthropology, Tohoku University Graduate School of Medicine, Sendai, Japan

Mesenchymal stem cells (MSCs) have been presumed to include a subpopulation of pluripotent-like cells as they differentiate not only into the same mesodermal-lineage cells but also into ectodermal- and endodermal-lineage cells and exert tissue regenerative effects in a wide variety of tissues. A novel type of pluripotent stem cell, Multilineage-differentiating stress enduring (Muse) cells, was recently discovered in mesenchymal tissues such as the bone marrow, adipose tissue, dermis and connective tissue of organs, as well as in cultured fibroblasts and bone marrow-MSCs. Muse cells are able to differentiate into all three germ layers from a single cell and to self-renew, and yet exhibit non-tumorigenic and low telomerase activities. They can migrate to and target damaged sites *in vivo*, spontaneously differentiate into cells compatible with the targeted tissue, and contribute to tissue repair. Thus, Muse cells may account for the wide variety of differentiation abilities and tissue repair effects that have been observed in MSCs. Muse cells are unique in that they are pluripotent stem cells that belong in the living body, and are thus assumed to play an important role in 'regenerative homeostasis' *in vivo*.

Key words: cell transplantation, mesenchymal stem cells, pluripotent stem cells, regenerative medicine, telomerase

Correspondence: Mari Dezawa, MD, PhD, Department of Stem Cell Biology and Histology & Department of Anatomy and Anthropology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan. Email: mdezawa@med.tohoku .ac.jp

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CURRENT STATE OF MESENCHYMAL STEM CELL (MSC) RESEARCH

Nearly 400 clinical studies of mesenchymal stem cell (MSC) transplantation have been performed around the world, targeting various diseases, such as Parkinson's disease, Crohn's disease, pulmonary fibrosis, and diabetes mellitus.[1–4] Sources of MSCs vary, with the bone marrow, adipose tissue, and umbilical cord currently being the most common. These sources are easily accessible and avoid the ethical problems associated with the use of fertilized eggs and fetal tissue. Tissue banks are available for bone marrow and umbilical cord tissues. Human MSCs have high proliferative activity and therefore large numbers of harvested MSCs can be obtained for clinical use.[5,6]

The most important requirement for clinical application is safety. To date, there have been no reports of tumorigenesis related to MSCs. They are not artificially induced or manipulated, but are naturally existing stem cells, and are thus considered non-tumorigenic. Although MSCs have great advantages for clinical use, they are not superior in all aspects, and the effects of MSCs on tissue regeneration and functional recovery are controversial.

While MSCs are referred to as 'stem cells', the rigorous methods of stem cell biology that are applied to hematopoietic and neural stem cells have not been applied in most of the studies performed using MSCs. Mesenchymal stem cells are usually collected just as adherent cells from the bone marrow and other mesenchymal tissues. While the morphology of collected adherent cells is similar to that of fibroblasts, they are not the same as fibroblasts. Some basic information about MSCs remains obscure, such as how many cells in the MSC population critically meet the criteria of stem cells, how many types of cells comprise MSCs, or the ratio of each cell type. The MSCs are a crude population and may include cells other than stem cells,

such as fibroblasts and endothelial cells, which are normally found in mesenchymal tissue.

Mesenchymal stem cells are heterogeneous, and their actions are pleiotropic. They produce humoral factors that exert trophic and anti-inflammatory effects and modulate immunologic reactions.[1,7] In addition to these humoral effects, MSCs exhibit a broad spectrum of differentiation abilities that cross the boundaries from mesodermal- to ectodermal- or to endodermal-lineage cells, suggesting that MSCs have an aspect of pluripotency.[8] Although at very low frequency, transplanted MSCs show triploblastic differentiation ability. In animal models transplanted with naive MSCs, the integration of a very small number of MSCs into damaged liver, brain, or heart, and differentiation into hepatocyte-, neural-, or cardiomyocyte-marker expressing cells in each organ were observed, suggesting the involvement of MSCs in tissue repair.[9–11] These tissue repair effects of MSCs, however, have not yet been clearly demonstrated in humans. Trophic effects are the most obvious effects of MSC transplantation, while tissue repair effects are considered to be minor and with a low frequency. Although MSCs are safe and feasible for clinical use, the low frequency of tissue repair effects limits the effectiveness of MSCs for regenerative medicine.

Nevertheless, MSCs are suggested to include a small population of stem cells that have the ability to differentiate into any cell type, much like pluripotent stem cells, and participate in tissue repair. Isolation of such stem cells from MSCs could have a critical impact in the fields of regenerative medicine and cell-based therapy. What kinds of cells might these be?

DISCOVERY OF MUSE CELLS

Pluripotent stem cells that account for one to several percent of MSCs, Muse cells, were first reported in 2010.[12] Muse cells are found in adult mesenchymal tissues such as the bone marrow, adipose tissue and dermis, but are generally distributed sparsely in organ connective tissue.[13] Muse cells can be conveniently obtained from commercially available mesenchymal cultured cells such as bone marrow- and adipose tissue-MSCs, as well as from fibroblasts, one of the most generally used cultured cells in the world, as several percentage of total MSCs. (Fig. 1)[12,14,15] Muse are pluripotent but non-tumorigenic, thus early realization of their application to regenerative medicine is highly anticipated.

The discovery of Muse cells is important in several aspects. First, the pluripotency of Muse cells and their small proportion of total MSCs are consistent with the previously reported low frequency of trans-differentiation of MSCs across triploblastic lineages. Second, the pleiotropic actions of MSCs are clarified by the division of the roles played by Muse cells and cells other than Muse cells, namely non-Muse cells. That is, Muse cells are responsible for the triploblastic differentiation and tissue repair effects, while non-Muse cells are deeply involved in trophic and immunosuppressive effects.[12,16]

Muse cells were initially identified as stress-tolerant cells. When bone marrow-MSCs (BM-MSCs) or fibroblasts are cultured for longer than overnight under stress-inducing conditions, e.g., incubated in trypsin or under low nutrition conditions, the vast majority of MSCs die and only a small number of cells, containing a high ratio of Muse cells, survive.[12] Somatic stem cells that normally reside in tissue are dormant and not usually active, but once the tissue is damaged or exposed to stress, they become activated and begin to proliferate, differentiate, and contribute to tissue restoration. In contrast to these stem cells, functioning differentiated cells tend to die after damage or stress. For example, neural stem cells that are located in the brain are normally inactive, but following stroke, these stem cells enter into the cell cycle and begin to generate neuronal and glial cells whereas mature neuronal cells tend to die.[17] Recently, Shigemoto *et al.* succeeded in efficiently collecting muscle stem cells, namely satellite cells, from adult skeletal muscle tissue by taking advantage of their stress tolerance properties.[18] In the same manner, Muse cells are stem cells that reside in mesenchymal tissues, and are tolerant to stress. In contrast to other somatic stem cells, however, such as neural and muscle stem cells, their actions are not confined to the tissue where they are located but they expand their field of activities, perhaps via the peripheral blood stream, and participate in extensive tissue repair, as described below.

CHARACTERISTICS OF MUSE CELLS

Muse cells have remarkable characteristics, including:

1. Muse cells are pluripotent stem cells that are able to differentiate into mesodermal-, ectodermal-, and endodermal-lineage cells from a single cell and can be directly collected from human tissues (Fig. 1).[12]
2. Muse cells can be obtained from easily accessed tissues, such as the bone marrow, adipose tissue, and dermis, as well as from commercially available cultured fibroblasts and BM-MSCs (Fig. 1).[12,13,15,16]
3. Muse cells have low telomerase activity and are non-turmorigenic.[14]
4. Muse cells comprise 0.03% of bone marrow mononucleated cells, and several percentage of cultured fibroblasts and BM-MSCs.[12]
5. Muse cells also comprise a part of MSCs, which are already used in clinical studies; thus, Muse cells are highly expected to be safe for clinical use.

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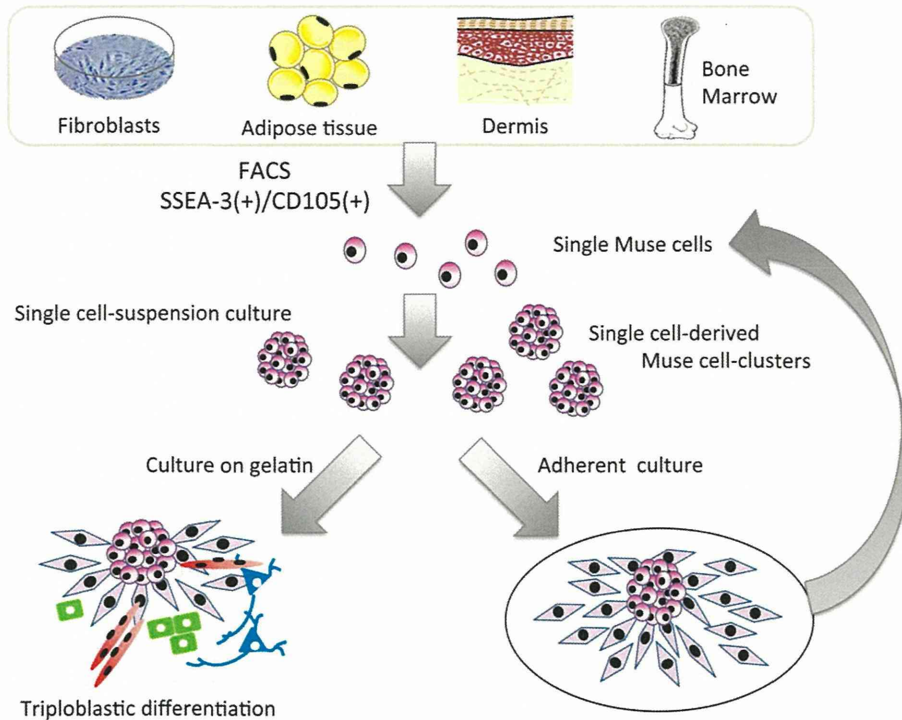

Figure 1 Pluripotency of Muse cells.
Muse cells can be collected from cultured mesenchymal cells (for example, fibroblasts and bone marrow mesenchymal stem cells (BM-MSCs)) and mesenchymal tissues (adipose tissue, dermis and bone marrow) as cells double-positive for SSEA-3 and CD105. After isolating Muse cells by fluorescence-activated cell sorting, single Muse cells cultured in suspension (single cell-suspension culture) generate characteristic clusters that are very similar to the embryoid bodies formed by human embryonic stem (ES) cells. When the cell clusters are transferred onto gelatin culture and spontaneous differentiation is induced, cells with endodermal- (i.e., hepatocytes), ectodermal- (neuronal cells), and mesodermal- (skeletal muscle cells) lineage are observed. The rest of the clusters were individually transferred to adherent culture and allowed to proliferate for 7 to 10 days, after which they underwent a second round of single cell-suspension in culture to generate second generation clusters. This experimental cycle was repeated three times, demonstrating that Muse cells maintain self-renewal, as well as triploblastic differentiation ability up to the third generation.

6 Muse cells have a proliferation rate of ~1.3 day/cell division, slightly slower than that of fibroblasts in adherent culture, so a large number of Muse cells can be prepared.[14]
7 Muse cells act as repair cells *in vivo*.[12]

Muse cells have dual aspects

Muse cells belong to MSCs. Therefore, they have nearly all of the properties of MSCs. Unlike general mesenchymal cells, however, Muse cells are pluripotent. These dual aspects of Muse cells are reflected by their expression of cell surface markers; they are positive for both mesenchymal (CD105, CD90 and CD29) and pluripotency (SSEA-3) markers (Fig. 1).[12]

Muse cells are unique, not only in their surface marker expression profile, but also in their behavior and other properties. In adherent culture, they appear similar to fibroblasts, but when they are transferred to a single cell-suspension culture, they can survive and begin to proliferate to form cell clusters that resemble embryonic stem (ES) cell-derived embryoid bodies formed in suspension. Such single cell-derived Muse cell clusters are similar to ES cells in their appearance, and positive for alkaline phosphatase as well as for the pluripotency markers Nanog, Oct3/4, and Sox2. Consistent with the expression of pluripotency markers, cells derived from Muse cell clusters are able to differentiate into endodermal-, ectodermal-, and mesodermal-lineage cells when transferred to gelatin cultures, proving that single Muse cells are able to generate cells representative of all three germ layers.[12,14] Importantly, non-Muse cells in MSCs have only mesenchymal aspects; that is, they do not express pluripotency markers, nor do they survive, proliferate, or form clusters in suspension.[12,14]

Triploblastic differentiation and self-renewal abilities of Muse cells

Muse cells are pluripotent stem cells because they can generate endodermal-, mesodermal- and ectodermal-lineage cells

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from a single cell and to self-renew (Fig. 1). The markers of each lineage into which Muse cells are able to differentiate are: ectodermal- (neural markers such as nestin, NeuroD, Musashi, neurofilament, microtubule associated protein-2, and markers for melanocytes such as tyrosinase, microphthalmia-associated transcription factor, gf100, tyrosinase-related protein 1, and dopachrome tautomerase[13,19]), mesodermal- (brachyury, Nkx2.5, smooth muscle actin, osteocalcin, oil red-(+) lipid droplets, and desmin[12,13]), and endodermal-lineages (GATA-6, α-fetoprotein, cytokeratin-7, and albumin[12,13]). Expression of these markers is recognized under both spontaneous differentiation on gelatin and cytokine induction systems.

With regard to ectodermal differentiation, Tsuchiyama *et al.* recently demonstrated that human dermal fibroblast-Muse cells could cross the boundary between mesodermal and ectodermal-lineages and efficiently differentiate into functional melanin-producing melanocytes by applying a cocktail of cytokines, including Wnt3a, stem cell factor, endothelin-3, and basic fibroblast growth factor, while the remainder of the fibroblasts, non-Muse cells, could not differentiate into melanocytes at all.[19] Muse cell-derived melanocytes expressed the melanocyte markers tyrosinase and microphthalmia-associated transcription factor, were positive for 3,4-dihydroxy-L-phenylalanine, an indicator of melanin production, and maintained their melanin-producing activity in the basal layer of the epidermis when transplanted into the skin. Together, these results demonstrated the absolute superiority of Muse cells over non-Muse cells in terms of pluripotency.

Although Muse cells are pluripotent, they tend to differentiate more frequently into their background lineage; they spontaneously differentiate into mesodermal-lineage cells with a higher percentage (10–15%) than into ectodermal (3–4%) or endodermal (3–4%)-lineage cells.[12]

The ratio of spontaneous differentiation of Muse cells is not very high, but an induction system with a certain combination of cytokines and trophic factors directs their differentiation more efficiently. For example, when Muse cells are treated with hepatocyte growth factor, fibroblast growth factor-4, and dexamethasone in insulin-transferrin-selenite medium, more than 90% of the cells become hepatocyte-like cells that express alpha-fetoprotein and human albumin[13] Muse cells treated with Neurobasal medium supplemented with B-27, basic fibroblast growth factor, and brain-derived neurotrophic factor differentiate into neuronal cells that are positive for MAP-2 and neurofilament.[13] In osteocyte or adipocyte induction medium, nearly 98% of Muse cells differentiate into cells positive for osteocalcin or oil-red, respectively.[13] In this manner, mesodermal-, ectodermal-, or endodermal-lineage cells can be more efficiently obtained from Muse cells, depending on the induction system. More importantly, none of the above differentiations requires the introduction of exogenous genes, and thus Muse cells produce the desired cells with lower risk.

Muse cells are self-renewable. When half of the first-generation clusters formed from Muse cells in single cell-suspension culture were transferred individually onto a gelatin culture and expanded, the expression of endodermal (alpha-fetoprotein, GATA-6), mesodermal (Nkx2.5), and ectodermal markers (MAP-2) was observed. The remaining clusters were individually transferred to an adherent culture and allowed to proliferate, after which they underwent a second round of single cell-suspension in culture to generate second generation clusters (Fig. 1). This experimental cycle was repeated up to three times and clusters from each step were analyzed. Expression of the above genes was detected in first, second, and third generation clusters, demonstrating that Muse cells maintain the gene expression profile required for self-renewal, as well as triploblastic differentiation ability.[16]

Non-tumorigenicity of Muse cells

When Muse cells are compared with tumoirgenic pluripotent stem cells such as ES and induced pluripotent stem (iPS) cells, the repertoire of the genes related to pluripotency, including Nanog, Oct3/4, and Sox2, expressed in Muse cells is similar to that of ES and iPS cells, while the expression level of those factors in Muse cells is lower compared to ES and iPS cells. Compatible with their tumorigenic activity, ES and iPS cells have high levels of telomerase activity as well as high expression levels of genes related to cell-cycle progression compared with Muse cells, which have the same low levels as naive fibroblasts.[13]

In sharp contrast with Muse cells, non-Muse cells do not originally express pluripotency genes. Expression levels of genes related to cell-cycle progression are similar between Muse and naive fibroblasts.[13]

Embryonic stem and iPS cells are known to form teratomas when transplanted *in vivo*. In fact, teratomas form when those cells are transplanted into the testes of immunodeficient mice.[12,20] In contrast, Muse cells do not develop into teratomas *in vivo*. Even after 6 months, none of the Muse cell-transplanted immunodeficient mouse testes formed teratomas (Fig. 2).[12,20] Together these results support that Muse cells are pluripotent but with non-tumorigenic and low telomerase activities. The non-tumorigenicity of Muse cells is considered to be consistent with the fact that they reside in normal adult mesenchymal tissue.

Ability of Muse cells to spontaneously repair damaged tissues *in vivo*

For application of ES and iPS cells to regenerative medicine in humans, two major conditions are required: (i) the cells must be differentiated into objective cells in a cell processing

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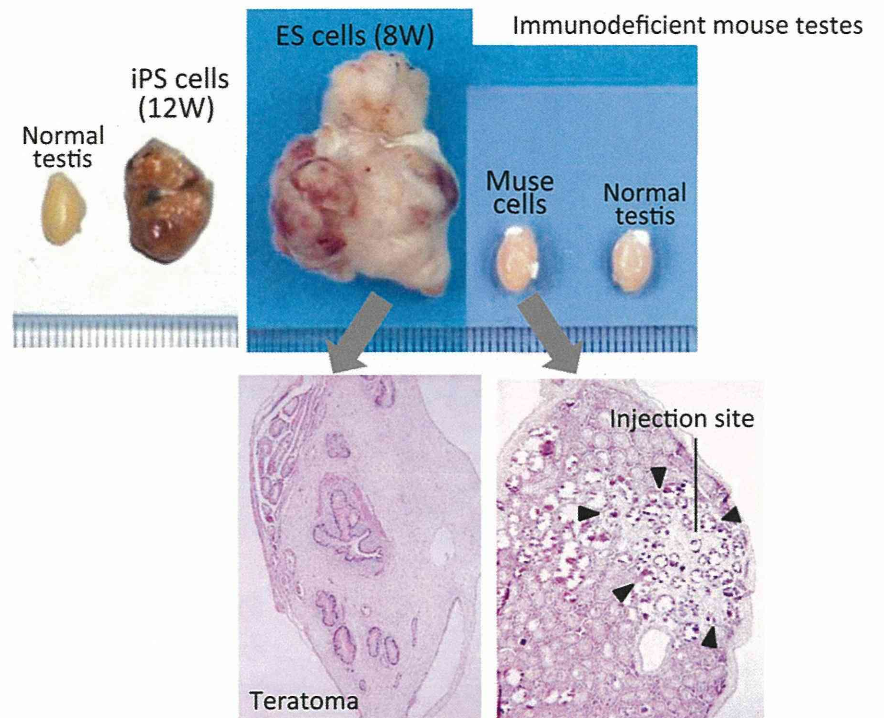

Figure 2 Muse cells are non-tumorigenic. When embryonic stem (ES) or induced pluripotent stem (iPS) cells were infused into immunodeficient mice (SCID mice) testes, they formed teratomas within 8 to 12 weeks while none of the Muse cell-transplanted testes generated teratomas and instead maintained normal tissue structure. (pictures reproduced from *Proc Natl Acad Sci USA* 2010; **107**: 8639–43 and *Proc Natl Acad Sci USA* 2011; **108**: 9875–80) (12,13).

center; and (ii) undifferentiated cells must be eliminated from the differentiated population before transplantation. These prerequisite conditions are based on the fact that undifferentiated ES and iPS cells have tumorigenic activity. As mentioned above, directly transplanted undifferentiated ES or iPS cells may form tumors *in vivo*. Furthermore, even if differentiation induction with high efficiency could be realized, some undifferentiated cells will remain.

For Muse cells, however, the above two conditions are not required. One possible scheme is that naive Muse cells can be applied directly to patients. Muse cells have the ability to migrate and integrate into the site of damage and then spontaneously differentiate into cells compatible with the tissue they target (Fig. 3). Such differentiation is observed in mesodermal, ectodermal, and endodermal tissues, and the Muse cells can act as 'repairing cells' in a wide spectrum of tissues and organs as described below.[12] Because differentiation and repair are induced spontaneously by Muse cells themselves, there is no need to control their differentiation prior to transplantation. Furthermore, as Muse cells are inherently non-tumorigenic and have low telomerase activity, it is not necessary to eliminate undifferentiated naive Muse cells. Ultimately, a cell processing center and complex systems are not necessary for Muse cell therapy.

The repairing effect of naive Muse cells is most striking in acute damage models. This was demonstrated by the infusion of green fluorescent protein-labeled naive human Muse cells into immunodeficient mouse (SCID mouse) models with fulminant hepatitis, skeletal muscle degeneration, spinal cord injury and skin injury. (Fig. 4)[12,21] Naive human Muse cells infused into the bloodstream of mouse models targeted damaged sites and differentiated into hepatocytes (positive for human albumin), skeletal muscle cells (human dystrophin), neuronal cells (neurofilament), and keratinocytes (cytokeratin 14), respectively (Fig. 4). The findings revealed that Muse cells can differentiate into ectodermal- (neuronal cell, keratinocytes), endodermal- (hepatocytes), and mesodermal-lineage cells (skeletal muscle cells) that are compatible with the targeted tissue and contribute directly to tissue repair.

While some infused Muse cells were trapped in the lung, the majority integrated into damaged tissues but not into intact tissues.[12] This suggests that disruption of blood vessels and destruction of tissues in damaged tissue are required for naive Muse cells to migrate and target, and thus Muse cells are able to perceive damage signals produced by damaged tissues. After integration, Muse cells differentiate into tissue-specific cells, but the factors that define the microenvironment of the site, which instruct the Muse cells how to differentiate correctly, remain unclear. Further elucidation of signals responsible for Muse cell migration and differentiation is needed.

DIFFERENT ROLES OF MUSE CELLS AND NON-MUSE CELLS IN MSCS

Although the action of MSCs is considered pleiotropic, recent findings of Muse cells are expected to elucidate the various

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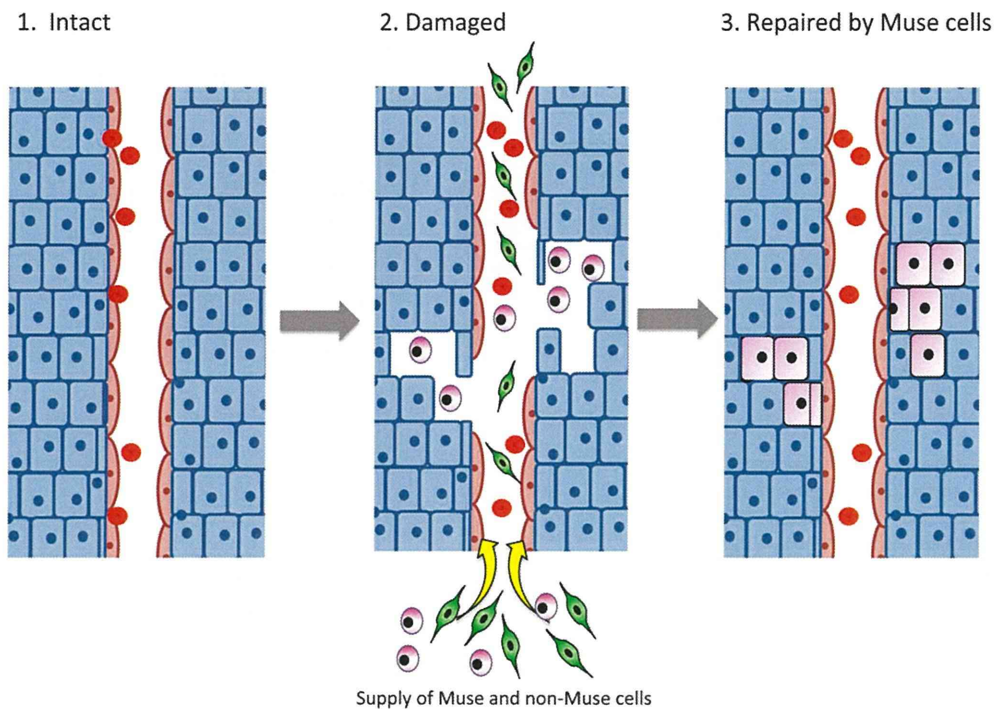

Figure 3 Tissue repair effect delivered by Muse cells. ●, red blood; ⦿, Muse cells; non-Muse cells.
When Muse and non-Muse cells were supplied to the blood stream, only Muse cells integrate into the damaged site, differentiate, and repaired the tissue, while non-Muse cells do not remain in the damaged tissue nor do they participate in tissue repair.

functions of the MSC components. Although Muse cells account for only several percent of the total MSCs, they play an exclusive role in triploblastic differentiation and tissue repair, while non-Muse cells do not directly participate in these events and rather have major roles in trophic and immunosuppressive effects. There are remarkable differences between Muse and non-Muse cells. First, non-Muse cells do not form clusters in suspension like single Muse cells.[12] Assuming that non-Muse cells are just like general mesenchymal cells, such as fibroblasts, they are essentially adherent cells and thus do not inherently survive and function in suspension.

Second, pluripotency genes that are expressed in Muse cells are not expressed in non-Muse cells and thus non-Muse cells are not pluripotent. Although they have lower efficiency than Muse cells, non-Muse cells do have the ability to differentiate into osteocytes, chondrocytes, and adipocytes. They are, however, unable to differentiate into neuronal cells (ectodermal), hepatocytes (endodermal), or even into the same mesodermal lineage skeletal muscles.[16] Thus, they are not pluripotent. Consistently, as shown in melanocyte induction, Muse cells from dermal fibroblasts can differentiate into functional melanocytes that produce melanin pigment following induction with cytokine cocktails while fibroblast-derived non-Muse cells fail to differentiate.[19] Gene expression patterns in non-Muse cells during melanocyte induction are interesting to observe; they respond partially to the induction stimulation and indeed some melanocyte markers are newly expressed in an earlier period of induction, but those markers disappear later and the gene expression pattern returns back to the original state of fibroblasts at the later stage.[19]

The partial responsiveness of non-Muse cells is also observed in iPS cell generation. Muse cells that are already pluripotent express pluripotency genes and lack tumorigenic activity, readily become iPS cells when treated with the four Yamanaka factors, and newly acquire tumorigenicity, whereas non-Muse cells do not show an increase in major pluripotency genes, including Nanog and Sox2, even after receiving the four Yamanaka factors.[13,22] Their responsiveness to the four Yamanaka factors is only partial, however, and thus non-Muse cells fail to generate iPS cells.

Third, non-Muse cells, unlike Muse cells, do not integrate nor differentiate into functional cells in damaged tissues.[12,21] Previous reports demonstrated that the large majority of MSCs do not remain in the transplanted tissue, but rather exert trophic effects that occasionally lead to some degree of functional recovery. As the majority of MSCs are non-Muse cells, the major role of non-Muse cells after transplantation might be a trophic effect.

LOCALIZATION OF MUSE CELLS *IN VIVO*

Mesenchymal tissues, such as the bone marrow, adipose tissue, and dermis, are the main reserve of Muse cells *in vivo*.

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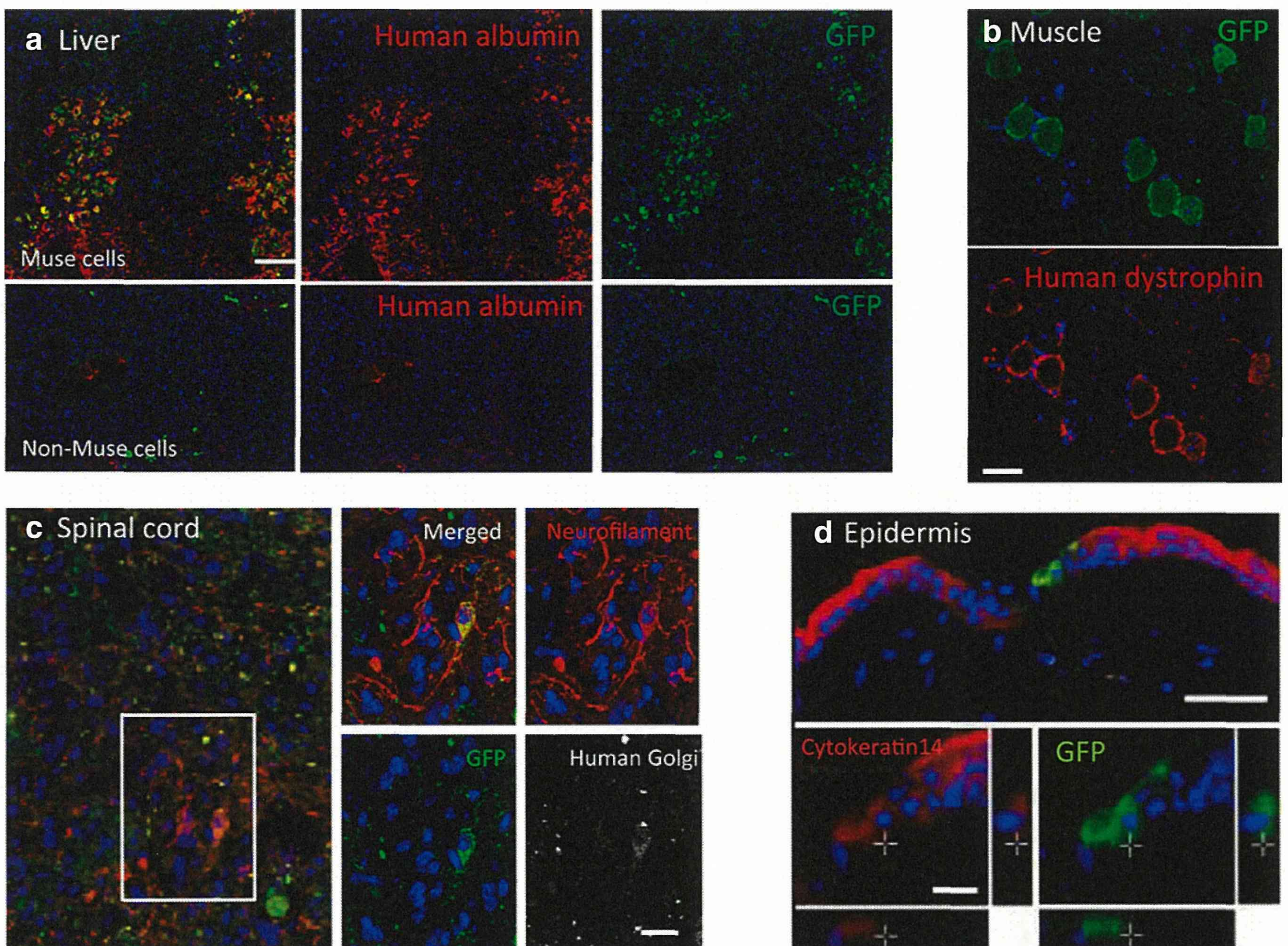

Figure 4 *Tissue repair effect of Muse cells.*
Green fluorescent protein (GFP)-positive human Muse cells integrated into (**a**) fulminant hepatitis, (**b**) muscle degeneration, (**c**) spinal cord injury (made by crush injury), and (**d**) skin injury models, and became (**a**) human albumin-, (**b**) human dystrophin-, (**c**) neurofilament- (cells were also positive for the human cell marker, anti-human Golgi complex, confirming that the positive cells were of human origin), and (**d**) cytokeratin 14- positive cells 4 weeks after injection. When non-Muse cells were infused into fulminant hepatitis, cells did not differentiate into albumin-positive cells. Scale bars; **a, b** = 100 μm, **c, d** = 50 μm. (Pictures reproduced from *Proc Natl Acad Sci USA* 2010; **107**: 8639–43, and *Cells* 2012; **1**: 1045–60, 2012).[12,21]

In the human dermis and adipose tissue, Muse cells detected as SSEA-3-positive cells locate sparsely in the connective tissues of the dermis and hypodermis, and do not associate with particular structures such as blood vessels or dermal papilla (Fig. 5)[13]. Similarly, they distribute in the connective tissue of many organs in the same manner as seen in the dermis and adipose tissue (unpublished data). Because tissue stem cells are generally confined to the tissue where the stem cells belong, i.e., neural stem cells in the brain, hematopoietic stem cells in the bone marrow, Muse cells are unique in that they are distributed throughout the body and are not confined to a specific organ or tissue.

Organ-derived Muse cells, however, might not be a practical source for clinical use. Rather, easily accessible mesenchymal tissues are realistic and feasible sources for obtaining Muse cells for clinical use. In the case of human bone marrow aspirate, SSEA-3/CD105 double-positive Muse cells were identified at a ratio of 0.03%, namely, 1 in 3000 mononucleated cells.[12] The proliferation speed of Muse cells is ~1.3 day/cell division, so that 10 ml of fresh bone marrow aspirate may yield nearly 1 million Muse cells within 10 days.[12]

Commercially available cultured mesenchymal cells, such as human dermal fibroblasts and BM-MSCs, are another potential source for Muse cells. While the ratio and quality of Muse cells may be altered by handling and depend the number of subcultures, fibroblasts and BM-MSCs contain Muse cells at levels ranging from 1% to 5–6%.[13]

MUSE CELLS AND REGENERATIVE HOMEOSTASIS

The fact that Muse cells reside in connective tissue and bone marrow suggests that they are widely distributed in the body.

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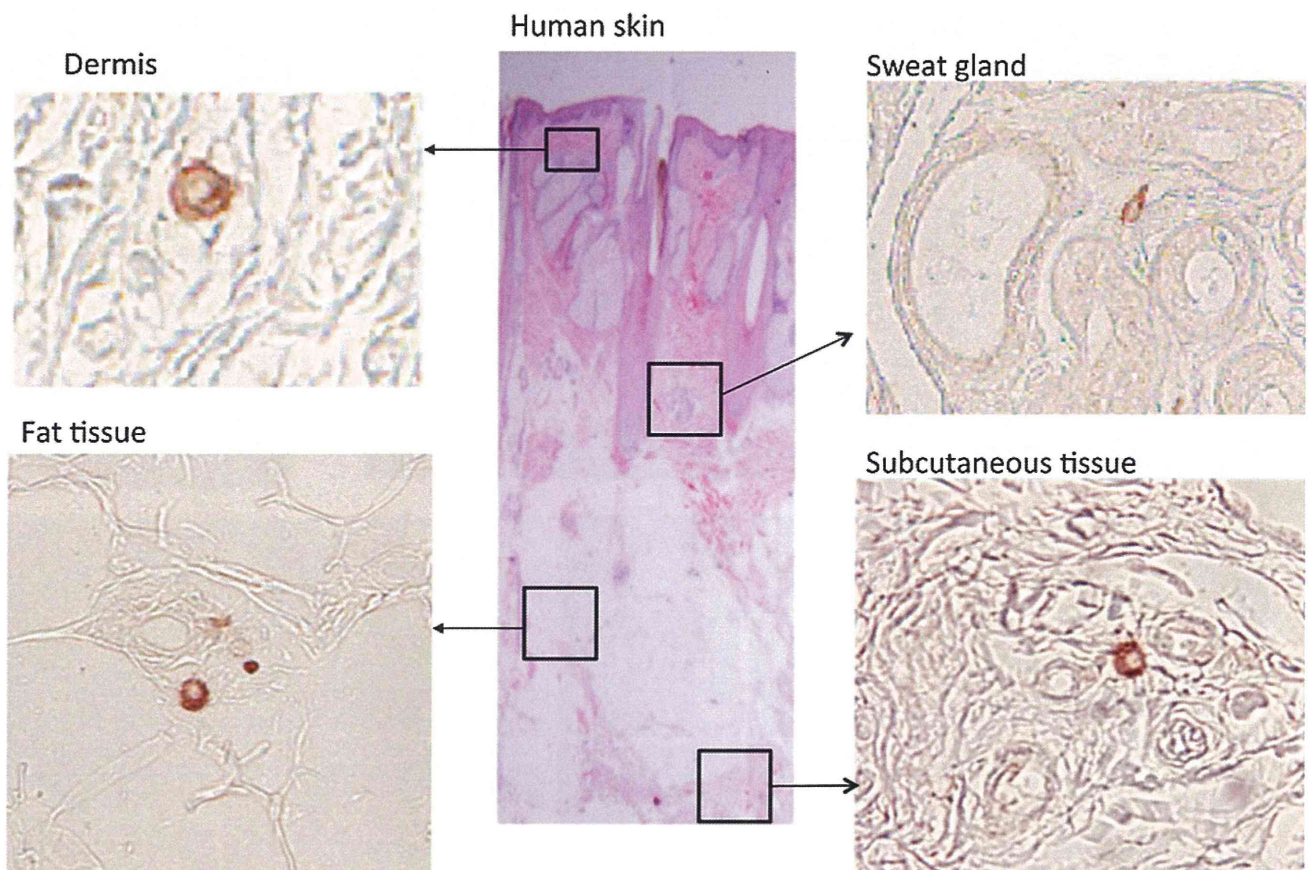

Figure 5 Muse cells sparsely locate in the adult human skin connective tissue.
Muse cells labeled by SSEA-3 are sparsely detected in the connective tissue of the dermis, sweat glands, adipose tissue and hypodermis. (pictures reproduced from *Proc Natl Acad Sci USA* 2011; **108**: 9875–80).[13]

If so, what kind of systems do Muse cells maintain *in vivo*? Because the bone marrow is directly connected to the peripheral bloodstream, the marrow is thought to be the hub of the Muse cell system in the body where the Muse cells are reserved and maintained in the normal state. Muse cells might be mobilized very slowly to the peripheral blood from the bone marrow in the normal state and distributed to the connective tissue of peripheral organs, including mesenchymal tissues such as adipose tissue and the dermis.

Comparison of the gene expression levels of Muse cells from bone marrow, adipose tissue, and dermis reveals that bone marrow-Muse express higher levels of genes related to ectodermal and endodermal-lineages than adipose- and dermal-Muse cells, suggesting that bone marrow-Muse cells have higher pluripotency than the other two types of Muse cells.[16] Bone marrow Muse cells are also unique in that they are highly dormant and more stress tolerant than adipose- and dermal-Muse cells.

Assuming that Muse cells build up a system *in vivo*, what is the function of Muse cells in the connective tissue of each organ? Because Muse cells are pluripotent, they can repair tissues that span endodermal-, mesodermal- and ectodermal-lineages. Connective tissue is very common and generally distributed in each organ, so that Muse cells residing in connective tissue can easily access small areas of damage that occur every day and replenish cells that are compatible with the tissue in the nearest parenchyma. It is conceivable that each organ is exposed to daily stress and minute damage that may cause cell degeneration. Our bodies are able to maintain function because of 'regenerative homeostasis' due to these small maintenance systems. The true mechanisms of regenerative homeostasis are still not clear, but the Muse cell system may have an important function. Further studies are needed to elucidate how Muse cells relate directly to regenerative homeostasis.

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