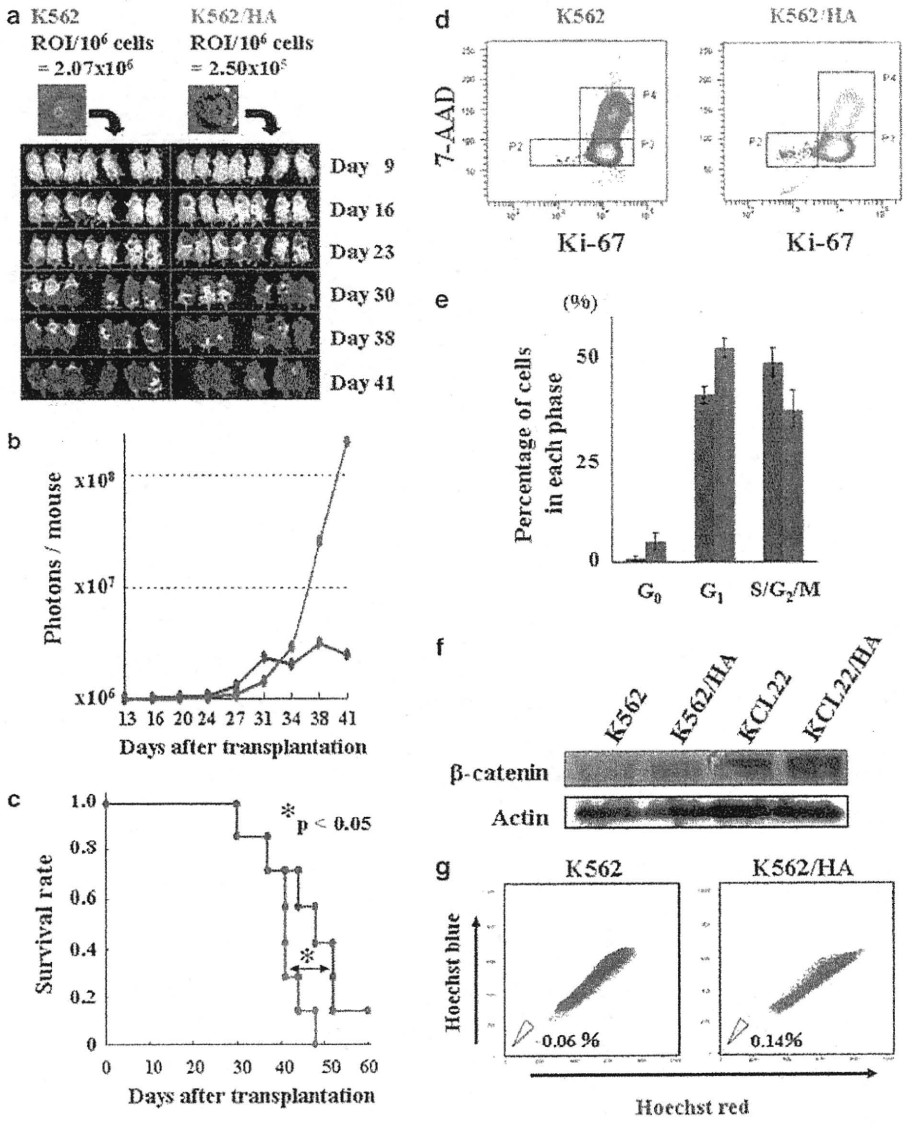

Figure 4 K562/hypoxia-adapted (HA) cells engraft more efficiently in NOD/SCID/γ_c^{null} (NOG) mice. (**a**) Whole animal experiments using NOG mice inoculated with $K562^{Luc\text{-}EGFP}$ or $K562/HA^{Luc\text{-}EGFP}$ cells. Engraftment was monitored by *in vivo* imaging. (**b**) Total photon emission from mice inoculated with $K562^{Luc\text{-}EGFP}$ (blue) or $K562/HA^{Luc\text{-}EGFP}$ (red) cells. (**c**) Survival rates of mice inoculated with $K562^{Luc\text{-}EGFP}$ (blue) or $K562/HA^{Luc\text{-}EGFP}$ (red) cells. (**d**) Cell cycle distributions of K562 and K562/HA determined by double staining with Ki-67 and 7-AAD. (**e**) The percentages of cells in each phase of the cell cycle in K562 (blue) and K562/HA (red), (**f**) β-catenin protein expression levels in K562, K562/HA, KCL22 and KCL22/HA cells. (**g**) Number of side population cells in K562 and K562/HA cells

Figures 8a–d) and mitochondrial outer membrane permeabilization (Supplementary Figures 8e–h). The proportion of cells undergoing apoptosis was greater in the BBGC- and COTC-treated K562/HA and KCL22/HA cells than in the parental cells (Supplementary Figures 9a–d). These results indicate that HA-CML cells were dependent on Glo-I activity for survival under hypoxic conditions, whereas the parental cells, cultured in normoxic conditions, were not dependent on Glo-I activity.

To evaluate the *in vivo* antileukemic activity of BBGC, we treated NOG mice inoculated with $K562^{Luc\text{-}EGFP}$ or $K562/HA^{Luc\text{-}EGFP}$ cells with BBGC. BBGC had no apparent effect on the survival of $K562^{Luc\text{-}EGFP}$-engrafted mice (Figure 5e). However, it significantly prolonged the survival of $K562/HA^{Luc\text{-}EGFP}$-transplanted mice (Figure 5f). The body weight of BBGC-treated mice did not decrease during the course of treatment (data not shown). These findings indicate that BBGC has potent antileukemic effects *in vivo*, and preferentially targets CML cells with higher Glo-I activity, with minimal associated toxicity.

Discussion

Abl TKIs can induce apoptosis only in actively proliferating Bcr-Abl$^+$ cells, making these drugs much less active against CML stem cells, which are predominantly in a quiescent

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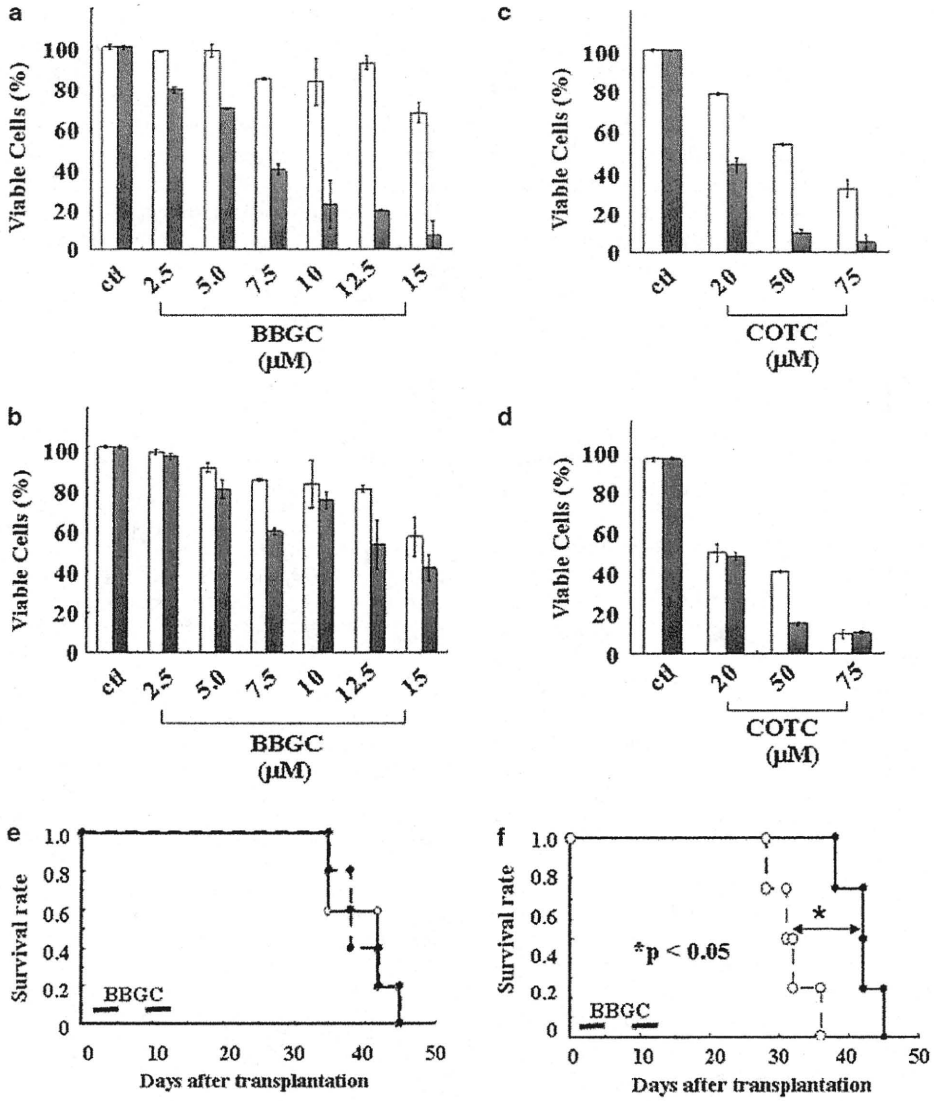

Figure 5 Effect of glyoxalase-I (Glo-I) inhibitors on hypoxia-adapted chronic myeloid leukemia (HA-CML) cells. *In vitro* cytotoxic effects of (**a**) *S-p*-bromobenzyl glutathione cyclopentyl diester (BBGC) and (**c**) 2-crotonyloxymethyl-4,5,6-trihydroxycylohex-2-enone (COTC) on parental K562 (white column) and K562/HA (black column) cells. *In vitro* cytotoxic effects of (**b**) BBGC and (**d**) COTC on parental KCL22 (white column) and KCL22/HA (black column) cells. *In vivo* effects of BBGC on transplanted (**e**) parental K562 and (**f**) K562/HA cells in NOD/SCID/γ_c^{null} (NOG) mice. Vehicle-treated mice, ○; BBGC-treated mice, ●

state.[10,11,29] Hypoxia favors the self-renewal of normal hematopoietic stem cells,[30] and resistance to hypoxia is one of the defining features of leukemic stem cells.[31] Therefore, it may be more important to search for new antileukemic agents that target quiescent CML stem cells residing in the hypoxic BM milieu. To this end, we have established two HA-CML sublines (Figures 2a–c). Previously, investigators have studied the role of hypoxia in leukemia using relatively short-term assays.[30–32] Our results suggest that transient hypoxia may not adequately mimic the physiological environment of CML cells (Figure 3e). Furthermore, in the mouse CML xenograft model, the oxygen concentration of engrafted leukemic cells in the BM was <10 mm Hg ($\sim 1.3\%$ O_2) (Figure 1f). Interestingly, the HA-CML cells exhibited characteristics similar to CML stem cells, including greater numbers of cells in a dormant, side population fraction, higher β-catenin expression, resistance to Abl TKIs and higher transplantation efficiency (Figures 2d, e and 4, Supplementary Figures 8 and 9).

The level of Bcr-Abl phosphorylation was lower in HA-CML cells, which may explain why those cells are less sensitive to Abl TKIs (Figure 2f). Giuntoli *et al.*[32] have also reported that the hypoxic selection of CML cells resulted in decreased cell sensitivity to imatinib and activation of Bcr-Abl-independent survival signaling pathways. Erk, a downstream effector of Bcr-Abl, was also less phosphorylated in HA-CML cells compared with parental cells, whereas the levels of p-Akt and p-Stat5 were similar (Figure 2f). These observations suggest that alternative mechanisms of activation exist for these signaling molecules in HA-CML cells.[33] In addition to

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the phosphorylation status of Bcr-Abl, the sensitivity to antileukemic agents depends on the balance of pro- and antiapoptotic molecules.[8] Akt pro-survival effects have been reported to be dependent on the first step in glycolysis.[34] However, Glo-1 induction in HA-CML cells was not directly controlled by Akt because Akt phosphorylation was unchanged in both HA cell lines (Figure 2f). The adaptation to hypoxia may also alter the status of Bcl-2 family members, because the expression levels of several proteins were altered in K562/HA cells. However, there were no changes in KCL22/HA cells. As the parental KCL22 cells are intrinsically resistant to imatinib and exhibit very high Bcl-2 expression, alterations in Bcl-2 family proteins may not be obvious. Further experiments will be required to clarify the hypoxia-induced changes.

We tried to identify a specific target in HA-CML cells that could be inhibited by small molecules with therapeutic potential. The dependence on glycolysis-mediated ATP production for uncontrolled cellular growth under limited O_2 conditions is a hallmark of malignant cells (Figure 3b).[35,36] Reduced ATP production (Figure 3a), as well as increased glucose consumption and lactate production (Supplementary Figure 4) in HA-CML cells, suggested that there was preferential utilization of glycolysis for ATP production in these cells. We focused on the components of glycolysis to identify leukemia cell targets that would circumvent drug resistance acquired through adaptation to hypoxia.[37,38] Glo-I is an enzyme that detoxifies methylglyoxal, a cytotoxic α-oxoaldehyde side product of glycolysis. Accumulation of methylglyoxal damages cells through multi-base DNA deletions and base-pair substitutions. The overexpression of Glo-I induces drug resistance to alkylating agents in leukemia as well as other solid tumors.[26,39] Glo-I activity was elevated in both K562/HA and KCL22/HA cells (Figures 3c and d), possibly because of their increased dependence on glycolysis during adaptation to hypoxia. Both K562/HA and KCL22/HA cells were more sensitive to cell killing by Glo-I inhibitors, indicating that these cells are indeed dependent on Glo-I activity for survival under hypoxic conditions (Figures 5a–d, Supplementary Figures 7b, c, 8 and 9).

All the Glo-I inhibitors examined were effective for killing HA-CML cells *in vitro*. We selected BBGC for *in vivo* analysis because BBGC was the most potent compound *in vitro*, and because BBGC has previously been used against lung cancer *in vivo*.[40] As engrafted K562 cells survive in the hypoxic environment of the BM (Figure 1f), we expected that BBGC would be effective against the parental $K562^{Luc\text{-}EGFP}$ cells, even if Glo-I activity in these cells was low. However, BBGC had no effect on the survival of $K562^{Luc\text{-}EGFP}$-engrafted mice (Figure 5e). This result may be due to the time differences between Glo-I induction by hypoxia and administration of BBGC. BBGC was administered from day 1 to day 11 after transplantation, whereas, in *in vitro* cultures, Glo-I was not induced until 21 days after the initiation of hypoxia (Figure 3e). Intriguingly, BBGC significantly prolonged the survival of $K562/HA^{Luc\text{-}EGFP}$-engrafted mice compared with untreated mice (Figure 5b). Thus, BBGC may be a promising therapeutic agent for use against CML cells with high Glo-I activity, which also more frequently accompanies the quiescent status. As the Abl inhibitor imatinib is currently the drug of choice for CML treatment, we examined the combined effects of BBGC with imatinib *in vitro*. BBGC augmented the effects of imatinib in killing CML cells *in vitro* (Supplementary Figure 10).

In conclusion, the survival of engrafted leukemic cells in the BM under severe hypoxia depends on the induction of Glo-I activity, and adaptation to hypoxia seems to result in the acquisition of Abl TKI resistance in CML cells. Glo-I inhibitors were much more effective against HA-CML cells than parental cells both *in vitro* and *in vivo*. These findings indicate the importance of the hypoxic environment for maintaining quiescent CML cells, and suggest that Glo-I is a novel target for CML treatment.

Materials and Methods

Reagents and cell lines. The Glo-I inhibitors, BBGC, COTC and methylgerfelin, were synthesized and purified as previously described.[26–28] The K562 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The other CML-derived cell lines (KCL22, BV173 and MYL) were kindly provided by Dr Tadashi Nagai (Jichi Medical School, Tochigi, Japan), Dr Oliver G Ottmann (Frankfurt University, Frankfurt, Germany) and Dr Hideo Tanaka (Hiroshima University, Hiroshima, Japan), respectively. The CML cell lines were maintained in RPMI-1640 supplemented with 10% fetal calf serum (Vitromex, Vilshofen, Germany) at 37°C in a humidified atmosphere of 20% O_2, 5% CO_2 and 75% N_2. CML cells were subjected to continuous culture in 1.0% O_2 (7.2 mm Hg), 5% CO_2 and 94% N_2, and HA subclones of K562 (K562/HA) and KCL22 (KCL22/HA) were selected and maintained in suspension in low O_2 conditions for more than 6 months. Parental K562 and K562/HA cells were cotransfected with pGL3, a control luciferase (Luc) reporter vector (Promega, Madison, WI, USA) and pCAG.*egfp.neo* using the Nucleofector Kit V, protocol T-03 (Amaxa AG, Cologne, Germany). Stable transfectants ($K562^{Luc\text{-}EGFP}$ and $K562/HA^{Luc\text{-}EGFP}$) were selected by culturing in medium containing G418 (1 mg/ml, Sigma Aldrich, Tokyo, Japan) and isolated by agarose gel cloning assays. Human primary Bcr-Abl$^+$ leukemic cells were obtained from patients with informed consent, according to the Declaration of Helsinki.

***In vivo* engraftment of CML cells and histological analysis.** Animal studies were performed in accordance with the guidelines of the Institutional Review Board for animal studies at Kyoto University. To evaluate the oxygen status of engrafted leukemic cells, 1.0×10^6 cells were injected into sublethally irradiated (2 Gy) male NOG or NOD/SCID mice at 6–8 weeks of age. At 35 days (NOG mice) or 50 days (NOD/SCID mice) post-inoculation, the mice were injected intraperitoneally with pimonidazole hydrochloride (Pimo, Chemicon, Temecula, CA, USA) (60 mg/kg), and 60 min later, the animals were killed and the femur and liver removed. Tissues were subjected to hematoxylin–eosin staining and immunohistochemical analysis using anti-Pimo (Chemicon) and antihuman Ki67 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies, and examined by microscopy. *In vivo*, leukemia cell proliferation was detected by monitoring luciferase expression using an *in vivo* imaging system (Xenogen, Berkeley, CA, USA), as previously described.[41] For survival analysis, time of death was determined either by spontaneous death, or by date of euthanasia due to pain or suffering, according to established criteria. We also confirmed the oxygen concentration of primary Bcr-Abl$^+$ leukemic cells that were obtained from a Ph$^+$ ALL patient and engrafted in the BM of NOD/SCID mice. Primary Bcr-Abl$^+$ leukemic cells (1×10^6) were transplanted into four NOD/SCID mice, which were killed 50 days after transplantation, and the BM sections were stained as above.

Cell death. Cell viability was measured by incorporation of propidium iodide (PI). Mitochondrial transmembrane potential ($\Delta\psi$m) was determined by staining with 3,3′-dihexyloxacarbocyanine iodide (Molecular Probes, Eugene, OR, USA), as previously described.[9] For analysis of DNA content, cells were fixed with ice-cold 70% ethanol and then incubated with PI as previously described.[42] The percentage of cells that incorporated PI, the $\Delta\psi$m, and the percentage of cells in sub-G_1 of the cell cycle were determined by FACS using the CellQuest software (Becton Dickinson, San Jose, CA, USA).

Western blot analysis. Proteins were separated by SDS-PAGE and then electroblotted onto a Hybond-PDVF membrane (Amersham Biosciences, Uppsala, Sweden). The membranes were incubated with 5% (wt/vol) nonfat dry milk in

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phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 (Sigma, Saint Louis, MO, USA). Antibodies specific for Akt (#9272, 60 kDa), Erk1/2 (#9102, 42, 44 kDa), phospho (p)-Akt (#9271, 60 kDa), p-Erk1/2 (#9101, 42, 44 kDa) and p-Stat5 (##9351, 90 kDa, Cell Signaling Technologies, Beverly, MA, USA); for Bcl-2 (clone 100, #05–729, 26 kDa) and p-tyrosine (#05-321, Upstate, Lake Placid, NY, USA); as well as for Stat5 (#sc-835, 92 kDa) and c-Abl (#sc-23, 120 kDa, Santa Cruz Biotechnology); Bcl-X_L (#AAM-080, 26 kDa, Stressgen, Victoria, British Columbia, Canada); Glo-I (#H00002739-A01, 28 kDa, Novus Bio, Littleton, CO, USA); β-actin (#A2066, 42 kDa, Sigma); Mcl-1L (38 kDa) and Mcl-1S (30 kDa) (#LS-C43163, Life Span Biosciences, Seattle, WA, USA); and for β-catenin (#610153, 92 kDa, BD Biosciences, San Diego, CA, USA) were used as indicated. Immunoreactive proteins were detected with horseradish peroxidase-conjugated secondary antibodies using enhanced chemiluminescence (ECL Advance, Amersham Biosciences).

Measurement of Glo-I activity. Cells were lysed in PBS containing 1 mM phenylmethylsulfonyl fluoride by freezing and thawing, followed by sonication. The lysates were centrifuged at 12 000 $\times$ *g* for 20 min and the supernatant was used as the cytosolic fraction. The Glo-I assay was performed in 0.1 M sodium phosphate (pH 7.0), 7.9 mM methylglyoxal (Sigma), 1 mM GSH and 14.6 mM $MgSO_4$ at 25 °C. An increase in absorbance at 240 nm because of the formation of *S*-D-lactoyl-glutathione was measured using a temperature-controlled spectrophotometer (Beckman Coulter$_A$ DU640).

ATP assay. ATP levels were measured using an ATP assay kit (TOYO INK, Tokyo, Japan), according to the manufacturer's instructions.

Measurement of glucose consumption and lactate production. Cells were suspended in fresh culture medium. After 6 h, the cells were collected by centrifugation and resuspended in 5 ml of RPMI at a density of 2×10^5cells/ml. Cells were incubated for 24 h, and the culture medium was collected for measurement. Glucose levels were determined using a glucose assay kit (GO, Sigma). Glucose consumption was determined from the difference in glucose concentration compared with the starting medium. Lactate levels were determined using a lactate assay (F-kit L-lactate, JK International).

Determination of quiescent cells. Numbers of quiescent leukemic cells (in G_0 phase) were determined by double staining with Ki-67 and 7-AAD as previously described.[43] Briefly, 1×10^6 K562 or K562/HA cells were fixed in ice-cold 70% EtOH for at least 12 h, and then resuspended in 100 μl PBS. Cells were stained with 20 μl Ki-67 antibody (BD Biosciences) and incubated for 30 min at RT. Subsequently, 20 μl 7-AAD (BD Biosciences) was added, and the cells were resuspended in 500 μl PBS with 1% FBS and analyzed by FACS. Three independent analyses were performed.

BBGC treatment of CML mice. NOG mice 6–8 weeks of age were sublethally irradiated (2 Gy) and inoculated with 1.0×10^6 K562/HA$^{Luc\text{-}EGFP}$ or K562/HA$^{Luc\text{-}EGFP}$ cells by intravenous tail vein injection. Therapeutic treatments (seven mice per group) were started 1 day (day 1) after transplantation. To prepare the BBGC solution, BBGC dissolved in cremophor EL/DMSO (1 : 1) was diluted to 10 mg/ml using DMSO and PBS. Within each group, half of the mice were administered EL/DMSO only (vehicle controls), and the remainder were administered 10 mg/kg BBGC on days 1 through 4, and days 8 through 11 after transplantation. For survival analysis, the time of death was determined either by spontaneous death or by date of euthanasia due to pain or suffering, according to established criteria.

Statistical analysis. Survival curves were drawn using the Kaplan–Meier method and compared using the log-rank test. *P*-values were derived from two-sided tests and a *P*-value <0.05 was considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions: M Takeuchi performed the research, analyzed the data and wrote the manuscript; S Kimura designed and performed the research, and wrote the manuscript; J Kuroda designed and performed the research, and wrote the manuscript; E Ashihara performed the research; M Kawatani performed the research; H Osada contributed vital new reagents; K Umezawa contributed vital new reagents; E Yasui performed the research; M Imoto contributed vital new reagents; T Tsuruo designed the research and contributed vital new reagents; A Yokota performed the research; R Tanaka performed the research; R Nagao performed the research; T Nakahata analyzed and interpreted the data; Y Fujiyama designed the research; T Maekawa designed the research and wrote the manuscript.

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Unique multipotent cells in adult human mesenchymal cell populations

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We found adult human stem cells that can generate, from a single cell, cells with the characteristics of the three germ layers. The cells are stress-tolerant and can be isolated from cultured skin fibroblasts or bone marrow stromal cells, or directly from bone marrow aspirates. These cells can self-renew; form characteristic cell clusters in suspension culture that express a set of genes associated with pluripotency; and can differentiate into endodermal, ectodermal, and mesodermal cells both in vitro and in vivo. When transplanted into immunodeficient mice by local or i.v. injection, the cells integrated into damaged skin, muscle, or liver and differentiated into cytokeratin 14-, dystrophin-, or albumin-positive cells in the respective tissues. Furthermore, they can be efficiently isolated as SSEA-3(+) cells. Unlike authentic ES cells, their proliferation activity is not very high and they do not form teratomas in immunodeficient mouse testes. Thus, nontumorigenic stem cells with the ability to generate the multiple cell types of the three germ layers can be obtained through easily accessible adult human mesenchymal cells without introducing exogenous genes. These unique cells will be beneficial for cell-based therapy and biomedical research.

bone marrow | differentiation | fibroblasts | mesenchymal stem cell | pluripotency

Recent advances in stem cell research have revealed the existence of various types of tissue stem cells that contribute to the functional maintenance of organs and to cell renewal, tissue remodeling, and repair (1, 2). These stem cells are expected to contribute to regenerative medicine, but this will require elucidation of their stem cell properties to control their proliferation and differentiation. Among the many kinds of tissue stem cells, hematopoietic stem cells and neural stem cells have been characterized most extensively (i.e., their ability to self-renew and differentiate into tissue-specific cell types has been clearly demonstrated at the single-cell level) (3, 4). In contrast, some of the properties of mesenchymal stem cells remain obscure. For example, one mesenchymal cell type, the bone marrow stromal cell (MSC), differentiates into cells of the same mesenchymal lineage, such as osteocytes, cartilage, and adipocytes, but also differentiates into cells of other lineages, such as neuronal cells and liver cells, suggesting that their differentiation is not tissue-specific, and that they are thus qualified as multipotent cells (5–8). In most cases, however, the differentiation was demonstrated in a heterogeneous population comprising MSCs and not at the single-cell level. Therefore, it remains under debate whether different subsets of cells are responsible for differentiation into cell types of different lineages, such as osteocytes and neuronal cells, or whether a distinctly multipotent stem cell type exists that is responsible for differentiation across all the oligo-lineage boundaries. Furthermore, hair follicle stem cells and dermal stem cells of the skin differentiate into cells positive for neuronal and smooth muscle cell markers, but their differentiation into cells of all three germ layers has not been demonstrated at the single-cell level (9–11).

In the present study, we demonstrate, at the single-cell level, that adult human skin fibroblasts, MSCs, and native bone marrow aspirates contain a distinct type of stem cell that is capable of generating cells with characteristics of all three germ layers. These cells are indistinguishable from other major mesenchymal cells in adherent culture, but when they are transferred to suspension culture, they form characteristic cell clusters that are positive for pluripotency markers and exhibit self-renewal and differentiation. Furthermore, they can be efficiently isolated as cells positive for both SSEA-3, a human pluripotency marker, and CD105, a mesenchymal cell marker. The cells exhibit multipotency, but their proliferation activity is not very high. Furthermore, although retaining their differentiation ability in vivo, these cells, unlike authentic ES cells, do not form teratomas in testes of immunodeficient mice. Our findings thus suggest that adult human mesenchymal cell populations, such as skin fibroblasts and MSCs, contain distinctly multipotent stem cells and that further studies of these cells will promote a better understanding of mesenchymal stem cell properties. Collection and enrichment of these cells should contribute to improved differentiation efficiency in mesenchymal cell populations. Finally, because these cells are easily accessible, they will be a realistic source of adult human multipotent stem cells that are capable of differentiation into cells with characteristics of all three germ layers without the need to introduce exogenous genes. These cells thus hold great promise for cell-based therapy and biomedical research.

Results

Analysis of Cell Clusters Generated from Human Mesenchymal Cells. We found that naive human MSCs (H-MSCs) grown in adherent culture spontaneously formed characteristic cell clusters at a very low frequency that appeared similar to clusters formed by human ES cells at an early stage (Fig. 1*A*) (12), suggesting that naive H-MSCs might contain multipotent cells. At a certain size, these cell clusters stopped growing and had a heterogeneous appearance (Fig.1*B*).

Dormant tissue stem cells are activated when tissues are exposed to stress, burdens, or damage (13–16). We therefore explored the possibility of whether stress conditions could be exploited for a method to enrich the putative stem cells in adult human mesenchymal cell populations. We subjected two strains of human skin fibroblasts (H-fibroblasts) and four strains of H-MSCs to six

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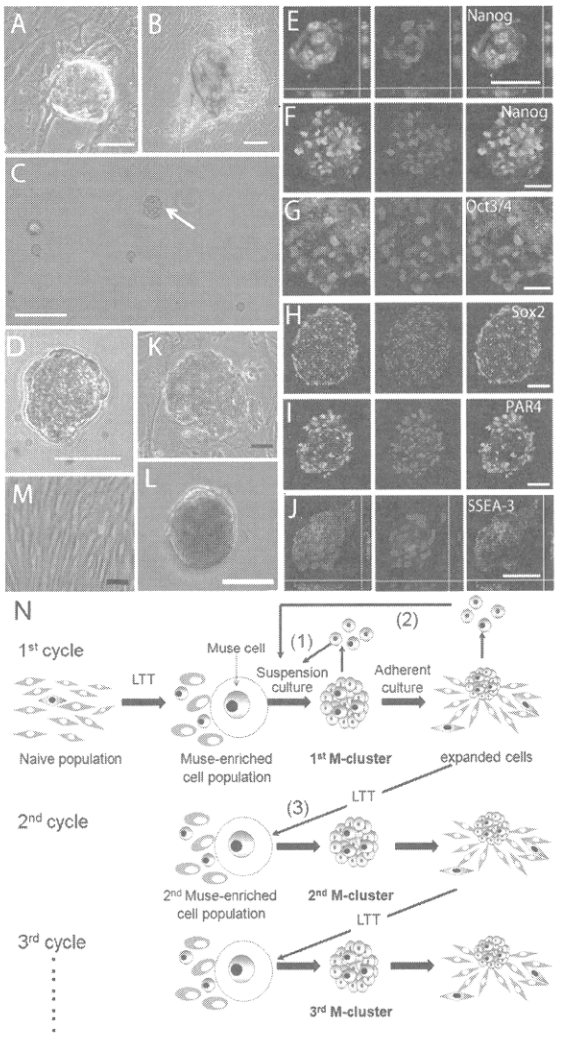

Fig. 1. Characterization of M-clusters. (*A* and *B*) Characteristic cell clusters that occur spontaneously in adherent cultures of naive H-MSCs. (*C* and *D*) MC culture of H-fibroblasts on day 7 showing an M-cluster (*C*, arrow). Immunocytochemical localization of Nanog (*E* and *F*), Oct3/4 (*G*), Sox2 (*H*), PAR4 (*I*), and SSEA-3 (*J*) in M-clusters formed by H-fibroblasts (*E*, *I*, and *J*) and H-MSCs (*F*, *G*, and *H*). ALP(+) human ES cells (*K*), M-cluster (H-fibroblast) (*L*), and naive H-fibroblasts (*M*). (*N*) Schematic diagram of the self-renewal of Muse cells. (Scale bars: *A–C*, 100 μm; *D–M*, 50 μm.)

different stress conditions, including long-term trypsin incubation (LTT) for 8 or 16 hr (Table S1 and *SI Materials and Methods*).

Stem cells are often grown in suspension culture, which is an efficient and convenient method to maintain their stem cell properties (17, 18). H-fibroblasts or H-MSCs that survived the stress treatments were therefore suspended in methylcellulose (MC) medium (19) at a density of 8,000 cells per milliliter (MC culture; *SI Materials and Methods*) and grown for 7 days (Fig. 1*C*). Each condition gave rise to cell clusters with sizes of up to 50–150 μm in diameter (Fig. 1*D*). Using different filters, we separated the cell clusters according to their size and characterized them by immunocytochemistry. Most of the clusters with a diameter larger than 25 μm contained cells positive for the pluripotency markers Nanog, Oct3/4, SSEA-3, PAR-4, and Sox2 and were positive for alkaline phosphatase (ALP) staining (Fig. S1). We therefore only counted cell clusters larger than 25 μm. Among the stress conditions tested, 16-hr LTT was most potent in the formation of cell clusters in H-fibroblasts, and 8-hr LTT was most potent in the formation of cell clusters in H-MSCs (Table S1 and *SI Results*). As expected, the formed clusters contained cells positive for the above pluripotency markers (Fig. 1 *E–J*) and ALP staining (Fig. 1 *K–M*). We called these cells multilineage differentiating stress enduring (Muse) cells because they express pluripotency markers; as described below, they differentiate into ectodermal, endodermal, and mesodermal cells; and they endure through stress conditions. We refer to H-fibroblasts and H-MSCs treated with 16-hr and 8-hr LTT, respectively, as "Muse-enriched cell populations" (MEC populations).

To calculate the frequency of Muse-cell-derived cell cluster (M-cluster; *SI Results*) formation accurately, MEC populations derived from both H-fibroblasts and H-MSCs were subjected to single-cell suspension culture after limiting dilution (Fig. S2 and *SI Materials and Methods*), showing that 11.6 ± 1.6% of the cells in the H-fibroblast–MEC population and 8.1 ± 0.2% of the cells in the H-MSC–MEC population proceeded to form M-clusters after 7 days. Naive populations (without LTT) were also examined and showed that 1.3 ± 0.1% (H-fibroblasts) and 1.1 ± 0.1% (H-MSCs) of the cells formed M-clusters in single-cell suspension culture after limiting dilution.

Self-Renewal and Expansion of Muse Cells. After LTT, Muse cells began to divide after 1–2 days in MC culture and continued to divide at a rate of ≈1.3 days per cell division until day 8, forming cell clusters. Cell proliferation gradually slowed by days 11–12 and ceased around day 14, with cell clusters reaching a maximum size of 150 μm (Fig. S3 and *SI Materials and Methods*).

When M-clusters formed in single-cell suspension culture after limiting dilution (day 12) were dissociated into single cells by a 5-min trypsin treatment and returned to single-cell suspension culture, the cells survived but divided very slowly (5–7 days per cell division) or sometimes not at all (Fig. 1*N1*). However, transfer of single M-clusters to adherent culture reinitiated cell proliferation and produced expanded cells. When cultures that had expanded to ≈3,000–5,000 cells were dissociated and subjected to single-cell suspension culture without LTT, 48.0 ± 5.8% (H-fibroblasts) and 40.3 ± 9.1% (H-MSCs) of the cells formed M-clusters (Fig. 1*N2*). When cultures were allowed to expand to $5–10 \times 10^4$ cells and were then subjected to LTT to produce MEC populations (Fig. 1*N3*), 12.3 ± 1.3% (H-fibroblasts) and 8.5 ± 0.5% (H-MSCs) of these cells formed second generation M-clusters. We repeated this culture cycle, consisting of LTT → suspension culture → adherent culture, five times, and every cell generation showed similar behavior and a similar frequency of M-cluster formation. We also confirmed that the fifth generation M-clusters were still positive for pluripotency markers and ALP staining. Furthermore, karyotypes of cells expanded from M-clusters did not show detectable abnormalities (Fig. S4 and *SI Results*). In conclusion, the proliferation activity of Muse cells is not very high, and proliferation stops in suspension culture when the cell clusters reach a defined size. Nevertheless, the proliferation of Muse cells can be reinitiated by transfer to adherent culture, which is followed by the formation of next-generation M-clusters, demonstrating the capacity of Muse cells for self-renewal and proliferation.

Differentiation of M-Clusters. To analyze their differentiation ability, single M-clusters formed in single-cell suspension culture after limiting dilution were transferred onto gelatin-coated dishes. After 7 days of culture, immunocytochemistry revealed cells positive for neurofilament-M [an ectodermal marker; the ratio of positive cells was 3.5 ± 0.5% (H-fibroblasts) and 3.7 ± 0.6% (H-MSCs)], α-smooth muscle actin [α-SMA; mesodermal, 12.2 ± 1.8% (H-fibroblasts) and 8.0 ± 0.6% (H-MSCs)], α-fetoprotein [endodermal, 2.7 ± 0.1% (H-fibroblasts) and 3.2 ± 0.3% (H-MSCs)], cytokeratin 7 [endodermal, 5.5 ± 0.1% (H-fibroblasts) and 3.4 ± 0.6% (H-MSCs)], or desmin [mesodermal, 14.2 ± 0.4% (H-fibroblasts) and 10.1 ± 0.5% (H-MSCs)] (Fig. 2*A–E*). RT-PCR of cells derived from first- and third-generation M-clusters confirmed that these cells

expressed α-fetoprotein (endodermal) and GATA6 (endodermal), microtubule-associated protein-2 (MAP-2; ectodermal), and Nkx2.5 (mesodermal), whereas these markers were not clearly detected in naive fibroblasts and MSC populations (Fig. 2*F*).

We injected MEC populations or M-clusters into the testes of immunodeficient mice to test whether they form teratomas. None of the testes injected with MEC populations or M-clusters formed teratomas for up to 6 months, and most of the testes were not significantly larger than control testes (Fig. 2*G* and *SI Results*). In the MEC- or M-cluster-injected testes, cells positive for human mitochondria and for ectodermal (neurofilament), endodermal (α-fetoprotein), and mesodermal (SMA) lineage markers were detected (Fig. 2 *H–M* and Fig. S5).

We next tested the differentiation of MEC populations in vivo by transplanting the cells into damaged tissues of the back skin (by local injection of GFP-labeled H-MSC–MEC population), gastrocnemius muscle (i.v. injection of GFP-H-fibroblast–MEC population), or liver (i.v. injection of GFP-H-fibroblast–MEC population) of immunodeficient mice. In regenerating skin, after 2 weeks, 79.5 ± 2.0% of the transplanted cells in the epidermis also expressed cytokeratin 14 (Fig. 3*A*). GFP(+) cells were also incorporated into regenerating muscle. After 2 weeks, the nuclei of these cells were centrally located, but after 4 weeks, 96.1 ± 2.2% of the GFP(+) cells had the appearance of mature myofibers with peripheral nuclei and expressed human dystrophin (Fig. 3 *B* and *C*). Some of the transplanted cells expressed satellite cell marker Pax7 (Fig. 3*B*). In the regenerating liver, after 4 weeks, most of the cells positive for human Golgi complex expressed human albumin (84.9 ± 5.3%) and human antitrypsin (87.6 ± 3.0%; Fig. 3 *D* and *E*). These data suggest that MEC populations can differentiate into ectodermal, endodermal, and mesodermal lineage cells in vivo.

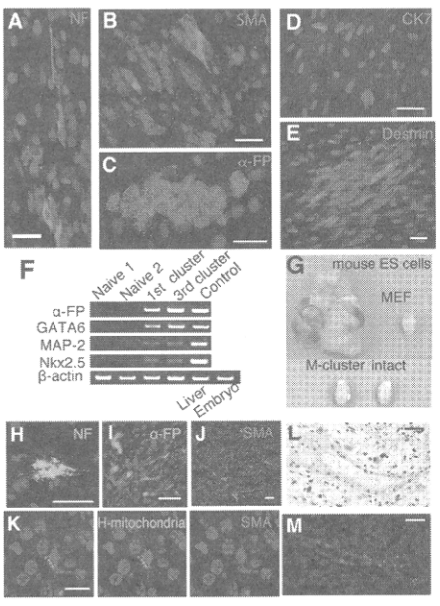

Fig. 2. Differentiation of Muse cells in vitro and in testes. Immunocytochemistry of neurofilament-M (NF) (*A*), α-SMA (*B*), α-fetoprotein (α-FP) (*C*), cytokeratin 7 (CK7) (*D*), and desmin (*E*) in cells derived from a single M-cluster (H-fibroblasts). (*F*) RT-PCR analysis of naive cells and first- and third-generation M-clusters (first and third clusters) derived from H-fibroblasts. Positive controls were human fetus liver (Liver) for α-FP and whole human embryo (Embryo) for GATA6, MAP-2, and Nkx2.5. (*G–M*) Testes of immunodeficient mice injected with cells. (*G*) Uninjected testes (intact) and testes injected with mouse ES cells (8 weeks), mouse embryonic fibroblast (MEF) cells (8 weeks), and M-clusters (6 months). Immunohistochemistry of NF (*H*), α-FP (*I*), and SMA (*J*) in testes injected with MEC populations and M-clusters. (*K*) Double-labeling of human mitochondria (green) and SMA (red). The tube-like structure (*L*) was positive for human mitochondria in the adjacent section (*M*; red). (Scale bars: *A–E* and *H–L*, 50 μm; *M*, 20 μm.)

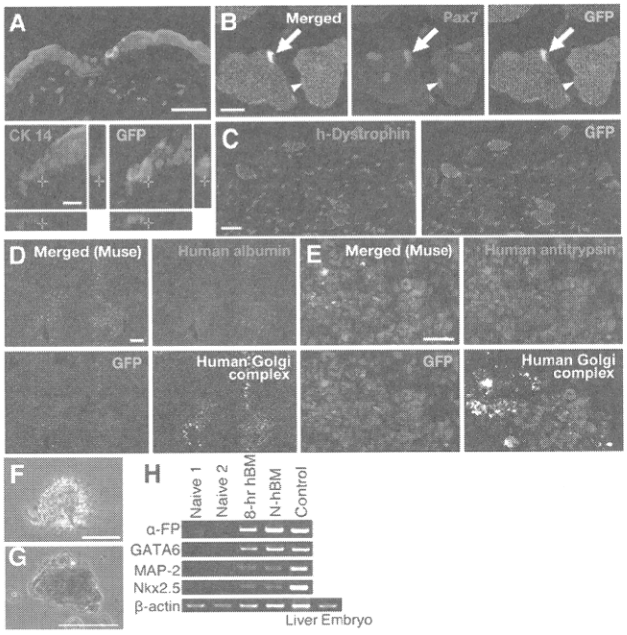

Fig. 3. Transplantation of Muse cells and M-cluster formation from bone marrow. (*A–E*) Differentiation of GFP-labeled MEC population (H-fibroblasts) in damaged tissues of immunodeficient mice. (*A*) Cells locally injected into the edge of the excised region. Transplanted GFP(+) cells expressed cytokeratin 14 (red) in the regenerating epidermis (2 weeks). (*B*) Two weeks after i.v. injection, GFP(+) cells with central nuclei were seen in cardiotoxin-injected cutaneous muscle. Transplanted GFP(+) cells (arrow) and host cells [GFP(−), arrowhead] that expressed Pax7 were seen. (*C*) After 4 weeks, the GFP(+) muscle fibers expressed human dystrophin (h-Dystrophin; red). Four weeks after i.v. injection, most of the transplanted GFP(+) cells in liver with CCl_4-induced damage were positive for human Golgi complex (*D* and *E*, white); some of them expressed human albumin (*D*, red) or human antitrypsin (*E*, red). (*F–H*) Formation of M-clusters from bone marrow-derived mononucleated cells. (*F*) M-clusters formed with 8-hr LTT (8-hr hBM-MC, day 7). (*G*) ALP(+) cells in 8-hr hBM-MC (day 7). (*H*) RT-PCR of naive H-MSCs (Naive 1 and Naive 2); M-clusters formed with 8-hr LTT (8-hr hBM) or without LTT [Naive hBM (N-hBM)]. Positive controls were human fetus liver (Liver) for α-fetoprotein (α-FP) and whole human embryo (Embryo) for GATA6, MAP-2, and Nkx2.5. (Scale bars: *A*, *B*, *E*, *F*, and *G*, 50 μm; *C* and *D*, 100 μm.)

M-Cluster Formation Directly from Native Bone Marrow Aspirate. The experiments described so far were performed with Muse cells and M-clusters derived from cell cultures, which may have acquired characteristics that differ from those of cells in situ. Mesenchymal cells are known to reside in the mononucleated cell fraction of bone marrow, which can be collected directly from native tissue without culturing. We therefore tested whether cells directly collected from human bone marrow (hBM) would also be able to form M-clusters. Isolated mononucleated cells were either subjected directly to MC culture (naive hBM-MC) or to 8-hr LTT before MC culture (8-hr hBM-MC). After 7 days, 8-hr hBM-MC formed M-clusters at a frequency of 0.3 ± 0.04%, ≈60–75 times higher than that of naive hBM-MC (0.004 ± 0.001%) (Fig. 3*F*). M-clusters from both naive hBM-MC and 8-hr hBM-MC were ALP(+) (Fig. 3*G*), and RT-PCR of cells expanded from single M-clusters on gelatin-coated dishes showed expression of α-fetoprotein, GATA6, MAP-2, and Nkx2.5 (Fig. 3*H*). These results suggest that M-clusters can be formed directly from hBM and that they can be enriched by 8-hr LTT.

Naive hBM-MC formed M-clusters at an extremely low frequency. Because culturing can change the composition of cell populations,

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cells in stable culture may have a different propensity to form M-clusters than cells from native tissues. To test this possibility, we grew hBM aspirate in adherent culture to collect primary MSCs and subjected the cells directly to MC culture without 8-hr LTT. This protocol resulted in a much higher frequency of M-cluster formation of 0.3 ± 0.08%. When primary MSCs were further cultured to the second and fifth passages, the frequency of M-cluster formation without LTT increased up to 0.5 ± 0.04% and 0.9 ± 0.1%, respectively. Consistent with this finding, 1.3 ± 0.1% of naive H-fibroblasts and 1.1 ± 0.1% of naive H-MSCs formed M-clusters in single-cell suspension culture as described above. These results suggest that Muse cells have a high stress tolerance and can endure in vitro culture and the subculture procedures.

Bone marrow contains many cell types, including MSCs, hematopoietic lineage cells, and endothelial cells (19). To determine which fraction contains the Muse cells, we isolated mononucleated cells from hBM aspirate and subjected them to magnetic affinity cell sorting (MACS) using antibodies against CD34 and CD117 [markers for hematopoietic cells (20)] and CD105 [marker for MSCs (5, 7)]. We then subjected the cells to 8-hr LTT and allowed them to grow in MC culture for 7 days. The $CD34^+/117^+/105^-$ cell fraction produced few M-clusters, but the $CD34^-/117^-/105^+$ fraction produced 50 times more M-clusters than the $CD34^-/117^-/105^-$ fraction (*SI Results*). This result suggests that the majority of Muse cells belong to the CD105(+) mesenchymal cell population.

Characteristic Features of Muse Cells. FACS analysis revealed that among tested surface markers, MEC populations showed a substantially increased number of cells that were SSEA-3(+) [a human pluripotency marker (12)] compared with naive populations (Fig. 4*A* and *SI Results*). The percentage of SSEA-3(+) cells detected by FACS [1.1 ± 0.05% (H-MSCs) and 1.8 ± 0.22% (H-fibroblasts); Fig. 4*A*] and immunocytochemistry [0.7 ± 0.1% (H-fibroblasts); Fig. 4*B*] in naive cells approached the previously determined frequency of M-cluster formation in single-cell suspension culture (≈1%), as described above. For MEC populations, the percentage of SSEA-3(+) cells detected by FACS [11.6 ± 0.15% (H-MSCs) and 8.6 ± 0.0.32% (H-fibroblasts); Fig. 4*A*] was also comparable to the frequency of M-cluster formation in single-cell suspension culture [11.6 ± 1.6% (H-fibroblasts) and 8.1 ± 0.2% (H-MSCs)] as stated. Furthermore, the expression of Oct3/4 and Sox2 in SSEA-3(+) cells was demonstrated in cultured H-fibroblasts using immunocytochemistry (Fig. 4 *D* and *E*). We therefore used FACS to separate SSEA-3(+) and (−) cells from MEC populations of both H-fibroblasts and H-MSCs and subjected each fraction to single-cell suspension culture after limiting dilution. The result showed that 56.5 ± 3.2% (H-MSCs) and 60.0 ± 4.5% (H-fibroblasts) of the SSEA-3(+) cells generated M-clusters, whereas only a few M-clusters developed in the SSEA-3(−) fraction.

The importance of SSEA-3(+) cells was also seen in the transplantation experiments. In contrast to the integration and differentiation of MEC populations in damaged skin, muscle, and liver (Fig. 3 *A–E*), transplantation of SSEA-3(−) populations resulted in a significantly smaller number of integrated cells and fewer cells that were positive for the tissue markers (Fig. S6 and *SI Results*).

Of note, at the scale of 3,000–5,000 cells, only ≈45.0 ± 3.2% (H-fibroblasts) of the cells that expanded from a single FACS-sorted SSEA-3(+) cell were SSEA-3(+) (Fig. 4*C*). This result suggests that proliferation of Muse cells may give rise to Muse cells and non-Muse cells.

The possibility remains that Muse cells are artificially induced by LTT. As described above, the majority of Muse cells exist in the bone marrow's CD105(+) cell fraction. Furthermore, SSEA-3(+) cells showed Muse cell properties. We therefore attempted to collect Muse cells directly from adult hBM aspirates by isolating them as SSEA-3/CD105 double-positive cells. Double-positive cells, which constituted 0.04 ± 0.008% of bone marrow-derived mononucleated cells, were directly subjected to RT-PCR, which showed the expression of Nanog, Oct3/4, and Sox2 in these cells (Fig. 4*F*). Isolated $SSEA\text{-}3^+/CD105^+$ cells were further subjected to single-cell suspension culture after limiting dilution without LTT. At 7 days, 11.4 ± 1.2% of the cells (corresponding to 0.003–0.005% of the mononucleated cells) formed M-clusters that were ALP(+). Single M-clusters were then again expanded in adherent culture to 3,000 cells and subsequently subjected to single-cell suspension culture. Of these cells, 33.5 ± 3.1% formed second-generation M-clusters, and RT-PCR of the cells that expanded from a single M-cluster on gelatin-coated dishes indicated that the cells expressed α-fetoprotein, GATA6, MAP-2, and Nkx2.5 (Fig. 4*G*), suggesting that cells with properties consistent with those of Muse cells reside in adult hBM.

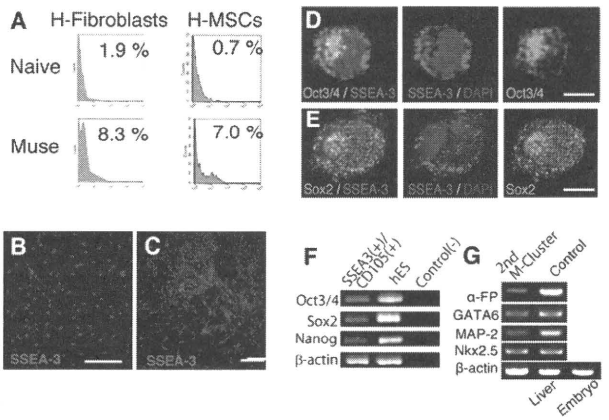

Fig. 4. Characterization of Muse cells. (*A*) FACS analysis for SSEA-3 expression in naive cells (Naive) and MEC populations (Muse) derived from H-fibroblasts and H-MSCs. SSEA-3(+) cells (red) in a naive population (*B*) and in cells expanded from a single M-cluster derived from a FACS-sorted SSEA-3(+) cell (*C*), both from H-fibroblasts. Immunocytochemistry of Oct3/4 (*D*, green, Sox2 (*E*, green), and SSEA-3 (*D* and *E*, red) in Muse cells derived from H-fibroblasts. (*F*) RT-PCR of Oct3/4, Sox2, and Nanog in directly isolated $SSEA\text{-}3^+/CD105^+$ cells from bone marrow, human ES cells for a positive control, and the template without reverse transcription for a negative control [Control(−)]. (*G*) RT-PCR of second-generation M-clusters (2nd M-cluster) from bone marrow-derived mononucleated cells. Positive controls were human fetus liver (Liver) for α-fetoprotein (α-FP) and whole human embryo (Embryo) for GATA6, MAP-2, and Nkx2.5. (Scale bars: *B* and *C*, 100 μm; *D*, 10 μm; *E–G*, 5 μm.)

Discussion

We isolated a specific type of human mesenchymal stem cell (i.e., Muse cell) that is capable of generating cells with the characteristics of all three germ layers from a single cell. Muse cells are (*i*) stress tolerant; (*ii*) indistinguishable from general mesenchymal cells in adhesion culture; (*iii*) able to form M-clusters in suspension culture that are positive for pluripotency markers and ALP staining; (*iv*) able to self-renew; (*v*) not very high in their proliferation activity and not shown to form teratomas in immunodeficient mouse testes; (*vi*) able to differentiate into endodermal, ectodermal, and mesodermal cells both in vitro and in vivo; and (*vii*) efficiently isolated from naive tissues as cells positive for both CD105 and SSEA-3.

To investigate whether Muse cells exist in native tissues or whether LTT induces cells to acquire properties of multipotent stem cells, we isolated SSEA-3/CD105 double-positive cells directly from hBM aspirates and subjected them to single-cell suspension culture without LTT. The formation of M-clusters showed that Muse cells or potential Muse cells already exist in adult native hBM. LTT is thus not necessary for collecting Muse cells but is a method to enrich them.

The properties of several kinds of stem cells present in mesenchymal tissue, such as neural crest-derived stem cells (NCSCs) that exist both in the bone marrow and skin, MSCs, skin-derived precursors (SKPs), perivascular cells, and adipose-derived stem

cells, were recently analyzed and described (11, 21–24). Of note, SKPs can be clonally expanded and serve as dermal stem cells for use in skin homeostasis and repair (11). NCSCs can also be clonally expanded, and they may contribute to nerve repair (22). Although, these stem cells can differentiate into ectodermal (e.g., neural marker-positive cells) and mesodermal (e.g., SMA-positive cells, osteocytes, chondrocytes) lineage cells (11, 21–24), their differentiation into representatives of all three germ layers has not been reported. Muse cells are unique among mesenchymal stem cells in that they are able to differentiate not only into ectodermal and mesodermal cells but into endodermal cells. That is, a single Muse cell can generate cells representative of each of the three germ layers. Considering that multilineage differentiation of MSCs, such as into neuronal cells, muscle cells, and liver cells, has been reported (5–8), it is possible that Muse cells contribute to such multilineage differentiation of MSCs.

Muse cells may have practical advantages for regenerative medicine, such as their easy accessibility and differentiation potential. They are not tumorigenic but retain differentiation ability in vivo after transplantation. In fact, they expressed endodermal, ectodermal, and mesodermal lineage markers after injection into mouse testes; integrated into damaged skin, muscle, and liver tissue; and differentiated into cells expressing the respective tissue markers cytokeratin 14 (ectodermal), dystrophin (mesodermal), and albumin (endodermal). Moreover, the multipotency of Muse cells does not need to be induced by the introduction of exogenous genes, and they can be isolated from skin and bone marrow, which are accessible from an individual or from a cell bank. Typically, each tissue contains a very small number of stem cells, and like other stem cells, the proportion of Muse cells in bone marrow-derived mononucleated cells is very small. However, large numbers of Muse cells can be obtained from mesenchymal cell populations by simply cycling the cells through a series of culturing steps, namely, Muse-cell selection followed by formation of M-clusters in suspension culture and expansion of the cells in adherent culture.

Muse cells did not show characteristics of tumorigenic proliferation, and, consistently, they did not develop into teratomas in mice testes. It has recently been reported that epiblast stem cells cultured under certain conditions also did not form teratomas in testes, even though they showed pluripotency in vitro (25). This finding suggests that even pluripotent cells do not always show the formation of teratomas in testes. Furthermore, if Muse cells are normally maintained in adult human tissues, such as in skin fibroblasts, their proliferation must be strictly regulated; otherwise, they would easily form tumors in virtually every part of the body.

Questions regarding the origin, location, and physiological roles of Muse cells in fibroblasts and MSCs will require further study.

Materials and Methods

Culture Cells. Two strains of human skin fibroblasts and four strains of H-MSCs were maintained at 37 °C in α-minimum essential medium (α-MEM) containing 10% (vol/vol) FBS and 0.1 mg/mL kanamycin. Mononucleated cells were collected from six hBM aspirates using Lymphoprep Tubes (Axis-Shield PoC AS). For selection of FBS, an H-MSC clone was plated onto a 24-well dish at a density of 1.5×10^4 cells per cm^2. Serum lots (ES cell grade; HyClone) were checked by adding a sample from each lot to a well at a concentration of 10% (vol/vol) in α-MEM and cultured for 1–2 weeks. The serum in the well that showed the highest frequency of spontaneous cell cluster formation, as shown in Fig. 1 *A* and *B*, was chosen for further experiments.

Generation of MEC Populations and M-Clusters. MEC populations were produced by treating cells with LTT (16 hr for H-fibroblasts and 8 hr for H-MSCs), followed by vortexing at 1,800–2,200 rpm for 3 min (MS1 Minishaker, IKA Works, Staufen, Germany) and centrifugation at 740 × *g* for 15 min. To produce M-clusters, individual cells were cultured in MC or in single-cell suspension culture. For MC culture, culture dishes were first coated with polyHEMA (P3932; Sigma) to avoid attachment of cells to the bottom of the dish. MC (MethoCult H4100; StemCell Technologies) was diluted in 20% (vol/vol) FBS in α-MEM to a final concentration of 0.9%. The cell concentration in the semisolid MC medium was adjusted to be 8×10^3 cells per milliliter. Cells and MC were mixed thoroughly by gentle pipetting, and the mixture was transferred to a polyHEMA-coated dish. At this concentration, the cell-to-cell distance was sufficiently large to minimize cell aggregation. For single-cell suspension culture, MEC populations were subjected to a limiting dilution with 10% (vol/vol) FBS in α-MEM and single cells were plated into each well coated with polyHEMA. The frequency of M-cluster formation was calculated from three experiments for each strain, with a minimum of 250 wells per experiment.

Detailed protocols for cell culture, stress conditions, ALP staining, immunocytochemistry, immunohistochemistry, transplantation experiments, RT-PCR, karyotyping, MACS sorting, and FACS analysis are provided in *SI Text*.

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第13章　再生医療分野における海外動向

1　アメリカFDAの提唱するphase I CGMPと日本の現状

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1.1　はじめに

細胞治療とは，輸血，造血幹細胞移植，細胞免疫療法などのヒト細胞を輸注，移植することにより行う治療法の総称である。再生治療や遺伝子治療の多くも，幹細胞を増幅させたり，分化させ機能を強化したりといった加工を受けた細胞を疾病の治療に用いようとするものである。この意味で，多くの再生治療や遺伝子治療は細胞治療として包括される（図1）。

細胞治療はあたらしい医療技術として，従来治療が困難であった疾病に対する革新的な医療技術として大いに期待されているが，その実現に向けて，細胞や組織の評価と安全性の確保が喫緊の課題である。わが国における一般的な医薬品や医療機器を開発するためには，薬事法に従い医薬品医療機器総合機構（Pharmaceutical and Medical Devices Agency：PMDA）へ治験計画を事

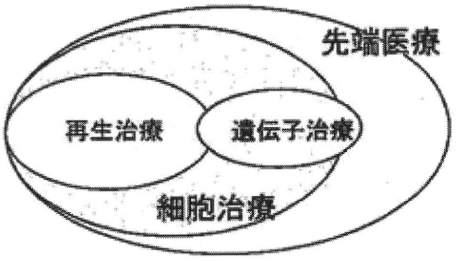

図1　細胞治療，再生治療，遺伝子治療，先端医療 ―言葉の定義―

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前に届け出て承認を受けて実施しなければならない。しかし，細胞治療に用いるヒト由来の組織や細胞は一般的な医薬品とは異なる特性を持つため，再生医療や細胞治療に用いる細胞の特殊性を理解して種々の規制を柔軟に適応させる必要がある。

米国において，第Ⅰ相臨床試験で使用される薬物や生物製剤を製造する場合には，連邦食品医薬品化粧品法（Food, Drug, and Cosmetic Act：FDCA）で要求されるCGMP（current good manufacturing practice，製造に関する基準）に従わなければならないとされてきた。第Ⅰ相試験に用いるための臨床研究新薬（治験薬）（Investigational New Drug：IND）の製造に対する規制は，被験者の安全性を保証することを主たる目的としている。さらに，米国食品医薬品局（Food and Drug Administration：FDA）は，CGMPにかかわる品質管理原則を臨床試験で用いる新薬の製造に適用することで，あたらしい治療法の開発が促進されるとともに，被験者保護につながると考えている。しかし米国でも，大学やベンチャー企業で行われている探索的臨床試験研究（トランスレーショナル・リサーチ，TR）に用いる研究用IND＊1の製造に，市販されている医薬品と同様の規制をかけていたのでは開発のスピードが上がらないことが懸念されるようになった。このようななかで米国FDAは，2006年1月にFDCAで要求されているCGMPに従った第Ⅰ相臨床試験のための薬品や生物製剤の製造を支援することを目的としたあたらしいガイダンス「INDs−Approaches to Complying with CGMP During Phase 1」の草案を公表した[1]。本稿では，このガイダンス（以下，Phase 1 GMPガイダンス）に基づいて米国FDAの提唱するCGMP準拠のアプローチの概要を解説するとともに，わが国の薬事法などの規制の現状を考慮し，著者らの考え方も適宜折り込みながら述べてみたい。したがって，上記のガイダンス原文の内容と異なる著者ら独自の見解も含まれていることをご理解いただきたい。Phase 1 GMPガイダンスの原文，あるいは著者らが邦訳したものを参照して頂ければ幸いである[2]。

1.2　背景

米国ではTRに用いる研究用INDの製造に対してFDAは柔軟な指導を行っている。1991年に，「（ヒトと動物の）研究用医薬品の準備におけるガイドライン」がFDAから交付された。しかし，このガイドラインは，規模の小さい，または実験室規模でのIND製造を含めたすべての製造状況について考慮しているわけではなかった。このような経緯を踏まえて，2006年1月にはFDCAで要求されているCGMPに従った第Ⅰ相臨床試験のための臨床研究新薬や生物製剤の製造を支援することを目的としたPhase 1 GMPガイダンスの草案がFDAから公表されたわけである。

＊1　本稿では探索的な臨床試験（TR）にもちいる臨床研究新薬（治験薬）を研究用INDと呼び，化合物のみでなくヒト細胞を含むものとする。なお，とくに治療用ヒト細胞のことに特化した記述の必要がある場合には，研究用IND（細胞）とした。

Phase 1 GMP ガイダンスでは，適切な品質の研究用 IND を得るために必要な管理とその程度は，それが探索的試験研究（TR）段階の製造である場合と，市販段階の製造である場合とで異なるべきであると言う考え方が提唱されている。すなわち，研究用 IND の製造と管理は，臨床試験の各相に応じて異なるべきであると言う考え方を示したものである（stepwise approach：図2）。FDA 当局が定めた CGMP に従えば，研究用 IND であっても，製造者には可能な限り，製品と製造目的，開発過程と製品情報，そして製造過程を反映するような管理方法を実行することが求められている[3]。

Phase 1 GMP ガイダンスは，「適用範囲」の項で説明されているとおり，第Ⅰ相臨床試験のために製造される研究用 IND について，その特殊な製造状況（例えば，研究室レベルのものであるか，探索的試験レベルのものであるのか，多品目を製造し多種類のロットを検査する必要があるのか），また特殊な製品（例えば，生物製剤／バイオテクノロジー製品，無菌処理製剤）における管理について，現時点での FDA の考え方を説明したものとなっている。Phase 1 GMP ガイダンスでは，第Ⅰ相臨床試験用に製造される探索的段階の製品には，従来の Part 211（21CFR211）において要求されている項目を全てにわたっては適用する必要がないとしている。ただし，研究用 IND がすでにスポンサー（臨床試験に対して責任を負い，これを主導する者*2）により，第Ⅱ相，第Ⅲ相の治験のために製造されたものである場合や，あるいはすでに合法的に医薬品として市販されているものである場合には，第Ⅰ相臨床試験で使用するものであっても，試験の規模や期間に関係なく，その製造は 21CFR Part211 項に従わねばならないとしている*3。

Phase 1 GMP ガイダンスは，最終決定されれば「適用範囲」の項で述べるような第Ⅰ相臨床試験用の研究用 IND 製造については，1991 年に FDA から出されたガイドラインに代わって適用されると考えられる。

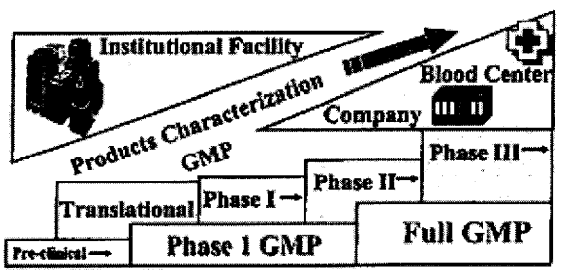

図2　Phase 1 GMP で提唱されている stepwise approach の概念

＊2　多くの場合は製薬企業。いわゆる医師主導型治験や臨床試験では，医師あるいは研究者を指す。

＊3　市販されている医薬品の適応外使用のことを指すと考えられる。

1.3　FDAの提唱するPhase 1 GMPの概要

1.3.1　適用範囲

Phase 1 GMPガイダンスは次のような研究に適用される。すなわち，第Ⅰ相臨床試験としてヒトに使用する研究用INDや生物由来製剤（プラセボとして投与される薬剤も含む），例えば，探索段階の組換え型または非組換え型治療用製品，ワクチン製剤，アレルギー製剤，生体内診断薬，血漿製剤，血液や血液成分，遺伝子療法製剤，体細胞療法剤（異種移植製剤を含む）などである。しかしながら，Phase 1 GMPガイダンスは以下のものには適用されないとしている。

・公衆保健サービス法（PHS Act）361項でのみ規制されるヒト由来の細胞や組織の製剤*4
・FDCAにおける医療機器承認規定の対象となる製品に対する臨床試験
・第Ⅱ相および第Ⅲ相臨床試験のために製造された研究用IND
・既に第Ⅰ相臨床試験における使用が承認された製剤（例えば，それを新たな適応のために使用する場合など）*5

1.3.2　法令準拠のための提言

Phase 1 GMPガイダンスは，INDの第Ⅰ相臨床試験を行うスポンサーと製造者がCGMPの要件に準拠するためにどのような方法をとるべきかについて述べている。製品開発の過程での研究用INDの品質や安全性は，適切な品質管理が有効に行われることによってある程度維持される。また，確立あるいは標準化された手順を用いることで，その後に続く第Ⅱ相，第Ⅲ相の臨床試験において必要とされる治験用製品と同等，またはそれに匹敵する研究用INDの製造が促されることになる。以下の要件を満たせば，おおむね第Ⅰ相臨床試験における品質管理手順を遵守することができると考えられる。

・明確に文書化された手順書（Standards Operating Procedure：SOP）
・適切に管理された施設
・検査または製造過程で得られた正確かつ一貫して記録されたデータ

製造者は，この3点以外に，Phase 1 GMPガイダンスで述べられている目標に見合った代替案を提案することもできる*6。INDが安全性，均一性，強さ，品質，純度の基準に見合ったものであることを保証するためには，スポンサーや製造業者が責任をもって，適切な方法を定め，設備を準備し，管理運用する必要がある。研究用INDの製造者は，特定の製品・製造業務に対

＊4　いわゆる"minimally manipulated"とされる。通常の治療法としてすでに確立している骨髄移植や末梢血幹細胞に用いる細胞などのことを指すと考えられる。

＊5　INDの適応外使用とでも言うべきものと考えられる。

＊6　製造者とFDAの間で行われるこのような議論（コミュニケーション）が，TRにおけるCGMPの適応を柔軟なものにしていると思われる。

するCGMPに適合した基準，教育訓練，作業手順の実施を保証する最適な方法を慎重に検討しなければならない。

1.3.3 Phase 1 GMPガイダンスとわが国の治験薬GMPに基づく規定との関係

Phase 1 GMPガイダンスをわが国の現状と比較すればどのようになるであろうか。Phase 1 GMPガイダンスにおける研究用INDの製造も，TRが主に行われる大学などの事情，TRの開発段階に伴うstepwise approachの必要性を考慮したものではあるが，わが国の現状に演繹して考えた場合，治験薬GMPにできるだけ準拠することを要求することになるであろう。実際，平成18年9月から施行された「ヒト幹細胞を用いる臨床研究に関する指針」では，細胞の調整は治験薬GMPに準拠することが要求されている[4]。

しかし，研究用IND（細胞）は通常の錠剤とは異なる細胞プロセシングと言う操作を必要とする。すなわち，錠剤などに適応される従来の治験薬GMPをそのまま研究用IND（細胞）に適応することは困難である。この議論に関連して，著者らは細胞治療用に特化したinstitutional GMP（iGMP）の必要性を提唱してきた[5～7]。表1にわが国における治験薬GMP，医薬品GMP，

表1　治験薬GMPと医薬品GMPの要求事項の相違点と，いわゆるiGMPの考え方

iGMP	治験薬GMP	医薬品GMP
同右（査察必要）	治験薬の製造に許可は不要	医薬品製造業の許可の要件
同右（iGMPの製造管理責任者は通常プロジェクト毎に異なり，常勤のものが行う）	品質管理者（製造管理者に相当）は薬剤師のほか，大学で薬学，医学，歯学，獣医学，理学又は工学を修め，必要な教育訓練を受けた者等でもよい。	製造管理者は薬剤師に限る。
同右（iGMPの品質管理責任者は人員面から各プロジェクトの兼務が基本になり，常勤のものが行う）	治験薬品質管理者は治験薬の品目ごとに置く。複数の治験薬についての兼務を妨げない。治験薬製造施設に常駐していなくてもよい。	製造管理者は製造所毎に置く。
同右	製造等の記録類の保管期間は，他の治験関係記録と同様の期間（5年間）。	製造等の記録類の保管期間は3年（生物10年，特生30年）
同右	他の試験検査機関等の利用は，治験薬品質管理者の判断に委ねられ，特に制限していない。	他の試験検査機関等の利用制限がある。
同右（細胞治療に特化したバリデーションが必須）	治験薬開発段階の目的に応じたバリデーションを実施すればよい。	多岐にわたるバリデーションが要求されている。
同右	委受託製造：治験薬の製造については許可は不要である。全部委託や再委託を妨げない。原薬等を含め，工程分断を妨げない。	委受託製造：委託側，受託側とも製造業の許可が必要。全部委託や再委託は認めない。原薬等の工程分断の禁止。
同右	製造施設：製造用水供給設備，試験検査設備については備えなくてもよい。	製造所：製造用水供給設備，試験検査設備が必要。
大学，研究所など	製薬企業，治験薬GMP製造受託会社	製薬企業

それにiGMPの相違点をまとめたが，iGMPの概念は，細胞プロセシングの特殊性，TRが主に行われる大学などの事情，それにTRの開発段階に伴うstepwise approachの必要性を考慮したものではあるが，基本的にiGMPは治験薬GMPと同様であり，「研究用IND（細胞）に特化した治験薬GMP」，すなわち「治験薬GMPの細胞版」と言う位置づけである。

治験薬GMPの目的は，① 治験薬の品質の均一性を保証することで臨床試験の信頼性を確保すること，② 治験薬と市販後製品の同一性を保証することで製品の有効性と安全性を確保すること，③ 治験薬の品質を保証することで不良な治験薬から被験者を保護することにある。治験薬GMPのハードの基準は，医薬品GMPに比べ，一部の設備については要求事項が緩和されている。これは医薬品と異なり，治験薬の製造においては，製造ロット数が少ないことや，治験の進行に伴い製造施設や設備が異なって行くことに対する配慮がなされているためである[8]。すなわち，米国FDAのPhase 1 GMPガイダンスで提唱されている研究用INDに関する"stepwise approach"の考え方を，わが国の治験薬GMPでは，概念的にではあるが，すでにとっていると言えよう。この意味で，Phase 1 GMPガイダンスにわが国の規制の枠組みを当てはめて考えれば，研究用INDは治験薬GMPに可及的準拠して製造することが望まれるわけである。

わが国においては，細胞治療や再生治療法に関するTRの場合，健康保険収載までを視野に入れた治療法としての確立を薬事法の枠内で目指す必要がある。すなわち，細胞プロセシングの特殊性を十分理解した上で，治験薬GMPの「運用」のなかで解決されるべきである。厚生労働省や総合機構（PMDA）も錠剤などの製造と細胞プロセシングは異なるものであることを十分認識しているし，如何にしてTRとしての在り方を，薬事法と言う現行の法律の枠組みのなかで構築し，推進させて行くのかが今後の大きな課題である。

1.3.4　Phase 1 GMPガイダンスの考え方をどのようにしてCGMPに適合させるか

多くの技術や既存のものを有効に組み合わせればCGMPに適合させることが容易になり，また製品開発を合理的に行うことができる。例えば，

・ディスポーサブルの設備や製造補助装置を使用すれば，清掃の負担を軽減できる。
・包装済みの注射用蒸留水や滅菌済み容器を使うことで，既存品を最適化するための付加的な設備や手順を省略できる。
・調整過程で製品が環境に暴露されない閉鎖系の設備を使うことで，作業室の空気清浄度を緩和できる。
・治験薬の製造を委託したり，その製品の試験検査を共有の施設や検査室を利用して行う。

などである。

「院内製剤」という概念のもとに，わが国においては，研究用INDの製造だけを目的として特別に設計されていない実験施設などで製造する場合も現時点ではあると考えられる。しかし，

Phase 1 GMP ガイダンスでは，このような場合，「研究用 IND の品質に悪影響を与える可能性のある製造環境が持つリスクに関して，スポンサーと製造者は十分に検討を加えなければならない」としている。すなわち，大学などで製造される研究用 IND も，原則的に CGMP に準拠することを求めているわけである。

以下にあげる内容について，Phase 1 GMP ガイダンスでは，製造者が特定の状況や用途に適した管理を導入できるように柔軟性をもたせている。

(1) 技術職員

わが国では，研究用 IND は製薬企業，ベンチャー企業，あるいは大学や研究所などの内部の施設において製造されていると思われる。企業はもちろんのこと，大学や研究所において，研究用 IND の製造にかかわるすべての技術職員または従業員，あるいは研究者は，職務を遂行するため十分な教育訓練を受け，品質管理の原則や CGMP での法的な要求事項を理解していなければならない。

(2) 品質管理

CGMP に従えば，すべての製造者がそれぞれ独自の品質管理 (Quality Control : QC) 計画を確立し，その計画を文書化する必要がある。例えば，QC 計画には以下にあげる事項が含まれていなければならない。

- 製品生産中に使用される様々な構成材 (容器，密閉容器，中間材料，包装素材，ラベル等) が，定められた品質基準に適合することを保証する文書 (分析証明書，Certificate of Analysis: COA など) の確認
- 製造手順，検査手順，承認基準の評価と承認に関する文書を作成し，実行させる責任の所在
- 臨床用の各ロットに対する完全な製造記録やその他の関連情報 (逸脱記録，試験・検査記録，承認基準に基づいた判定結果など) の累積評価に基づいた出荷の合否判定に必要な文書の作成と，その判定に対する責任の所在
- 製造中に予期せぬ結果や過失が生じた場合の調査と，是正処置の開始に必要な文書の作成と報告を遵守する責任の所在

また，QC 責任と製造責任とは当然独立して実施されねばならない。すでに承認され市販されている医薬品の場合，製造に関する試験検査などの業務は通常専任の品質管理担当者によって行われている。しかし，大学などで研究用 IND を製造する場合，組織の規模や構造によっては，限られた状況下で，すべての品質管理業務が同一の人物によって行われている可能性もある。また，ベンチャー企業や大学内の施設などでは，製造作業と各ロットに対する出荷の合否判定を含めた品質管理が同一人物によってなされるといったことも行わざるを得ない場合があると考えられる。このような状況下であっても，QC 責任と製造責任を独立させるために，製造業務に関わ

らない別の人物が製造記録の付加的，定期的な評価を行う品質管理者として権限を与えられるべきである。

(3) 設備および装置

第Ⅰ相臨床試験に用いられる研究用 IND を製造するために使用される施設はすべて，たとえそれが大学や研究所における施設（研究室）であっても，以下にあげるような目的の作業に適した作業場所と設備を有するべきである。

・充分なスペース，清潔な環境（クリーンルーム），適切な建造物
・適切な照明，換気，冷暖房設備
・適切な配管，洗浄，衛生設備
・汚染，交差汚染を防止するための適切な空気処理システム（層流式フード等）
・製品を汚染させず，製品に対する反応性，付着性，吸収性を持たず，正しく保管され，後述する手順に従い定期的に校正，清掃，衛生管理された設備

個々の工程で使用される設備はすべて特定し，製造記録に記録を残すことが推奨される。また，無菌処理を伴う研究用 IND（細胞）については，後述する「無菌製品および無菌処理製剤」に従うことが推奨される。適切な施設において手順を管理することが，製品汚染や交差汚染，取り違えを防止するのに役立つ。

(4) 成分材料の管理

研究用 IND の製造段階で使用される成分材料の取扱い，評価，承認や管理に関して手順書（SOP）を作成することが推奨される。成分材料は，検査や試験が終了して製品製造のために出荷承認されるまでは他の材料から隔離しラベル表示などを行い保管管理されなければならない。また，成分材料の品質劣化や汚染防止のために個々の特性に則した基準を設け，保管や取り扱うことが重要である。すべての成分材料の関連情報を含む記録を残すことも必要であり，記録として残しておくべき事項としては，納品日，出荷用量，供給者の名前，成分材料のロット番号，研究用 IND のロット番号，保管状態，使用期限などが挙げられる。

成分材料がその属性について設けられた承認基準に適合していることを保証するために，成分材料のロット毎の分析証明書（COA）またはその他の証明書を入手して確認することが推奨される。ヒト由来や動物由来など特定の原材料に対する証明書には，それらの供給元に関する情報あるいは感染症に関する検査結果が含まれるべきである。構成成分に対する証明書が不十分である場合には，不十分であった内容に関して検査することが望ましい。

(5) 製造と文書化

研究用 IND の製造は，以下の条項を含む製造手順書（SOP）に従って行うべきである。

・検査結果の記録や使用される成分材料，器具，操作手順などについての詳細な製造記録を保管

する。スポンサーは製造工程を忠実に再現するために必要な手順をすべて文書化し，この書類を保管管理する。また，研究用 IND の製造が中断された場合には製造中断の理由を記載した記録書を残す。

・製造が中断された場合には，適用した処理手順の記録を残す。また，製造行程の変更が必要な場合には，変更に関するすべての論理的根拠と変更記録を残す。

・Phase 1 GMP ガイダンスの対象となる無菌処理の必要な研究用 IND（細胞）の製造に適応される文書化された手順と微生物学的管理記録を保管管理する。無菌操作の技術と，微生物やエンドトキシンなどによる汚染の防止を目的とした中間材料の管理を行う（「無菌製品および無菌処理製剤」の項を参照）。

(6) 検査室の管理

① 検査

製造工程の途中で行われる検査は一定の条件で管理され，文書化された検査手順書に従って実施されることが推奨される。研究用 IND の均一性，強度，効力，純度，品質などといった属性を評価する検査が適切に実施されなければならない。既に知られている安全性に関する事項については，個々の製品がそれに適合していることを確認する。しかし，製品によっては製品開発のこの段階においては適切な承認基準がすべて満たされるとは限らないが，その内容は研究用 IND 申請時に審査されることになる。

検査室で使用される測定機器は検査結果の信頼性を確保するため，常に適切な間隔で校正を実施する必要があり，文書化された手順に従って管理されるべきである（機器のバリデーションの必要性）。作業者が試料の分析や設備の適合性などの検査を行う際には，検査装置の稼働状態が正常であることを事前に確認する。

さらに製造ロット毎の代表サンプルを保管管理する際には，可能であれば出荷検査を行うのに必要な量の 2 倍量のサンプルを保存する。サンプルは臨床試験終了後または IND 申請取り下げ後，少なくとも 2 年間は適切に保管されるべきである。

② 安定性

スポンサーは，臨床試験の期間中に製品の安定性と品質を監視するために，研究用 IND の代表サンプルを使って安定性の検査を開始することが推奨される。

(7) 梱包とラベル添付

Phase 1 GMP ガイダンスの対象となる研究用 IND が保管中，あるいは出荷される場合，保管，取扱い，輸送の間に変性，汚染，損傷がないよう適切に梱包しなければならない。また，取り違いを防ぐためにラベルの添付と保管作業の整備が推奨される。

(8) 配給

第Ⅰ相臨床試験に関する限り，「配給」という言葉は，Phase 1 GMP ガイダンスの対象となる試験段階の新製品（研究用 IND）が臨床研究者（医師）に，そして最終的には，その研究に登録されている被験者に投与されるまでを含んでいる。製品の品質を保証するために，製品はラベルに表示された保管条件に従って扱われるべきである。Phase 1 GMP ガイダンスの対象となるロット毎の研究用 IND 配給記録により，追跡調査ができ，必要に応じて製品の回収を容易にとなるので，正確に記録されなければならない。

(9) 記録保管

スポンサーは以下の要項を含む品質と製造工程での作業に関する完全な記録を保管すること。

・設備・機器の保守点検および校正記録
・製造記録および関連する分析検査の結果
・配給記録
・すべての品質管理項目
・成分材料の記録

IND 規則の下では，医薬品として承認され，新薬申請書（New Drug Application：NDA）が受理された後少なくとも 2 年間，あるいは NDA として承認されなくても，臨床試験に使用するための研究用 IND の出荷および配給が中止され，FDA にその旨が報告されてから 2 年間，スポンサーは記録を保管しなければならない。

1.3.5　特殊な製造状況

(1) 複数の研究用 IND 製造に使用される（多種類の研究用 IND を製造する）施設

ある製品が生産される区域や作業室は他の作業から隔離されていることが理想的である。しかし，適切な清掃や管理手続が的確に実施され，材料や製品の残留や混同がないことが保証されていれば，同じ区域や作業室が他の試験製品の製造あるいは検査を含む多目的に利用することは可能である。この場合，作業区域の設計や配置は材料や器具などが整然と配置されており，順序立てて取り扱うことができ，取り間違いの防止，以前に製造された製品などの混入，あるいは作業員や環境からの汚染防止に配慮されていることが推奨される。

研究用 IND の製造のために使用される試薬や成分材料には適切にラベルを貼り，取り違えや誤った使用を避けられる方法で整然と整理していれば，研究のために使用されるものと同じ区域内でも安全に保管できるであろう*7。ただし，一つの試薬や成分材料はどの時点においても，

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*7　研究用 IND の製造を通常の実験室や研究室で行っても良いと言う意味ではない。試薬や成分材料の保管に関する記述であり，実際には，同じ区域内と言っても研究用 IND 専用の保管庫が必要である。できれば，研究用 IND 専用の保管区域を設けることが望ましい。