Identification of human bone marrow-derived long-term mesenchymal stem cells

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Disclosures: The authors declare they have no conflicts of interest.

INTRODUCTION: Human bone marrow-derived mesenchymal stem cells (MSCs) are a heterogeneous population with distinct proliferative and differentiation potentials. Aging was associated with impaired MSC function, contributing to reduced tissue regeneration and the pathogenesis of bone and cartilage disorders. However, the functional and transcriptional heterogeneity of human MSC subclusters and their age-dependent alterations remain incompletely understood.

METHODS: Twenty-eight human samples (6 males and 22 females) were obtained as surgical waste from total hip arthroplasty. The study was conducted in accordance with the World Medical Association Declaration of Helsinki, and all experiments were approved by and performed under the guidance of the Kobe University Graduate School of Medicine Ethics Committee. MSCs were isolated as $CD45^{-}CD271^{+}$ populations from femur head bone marrow of middle-aged and elderly donors. The age range of the patients who were included in the study was 36-89 years (Middle-aged group: 36-65 years (n=18); Aged group: 72-89 years (n=10)). Bone marrows were harvested and crushed with rongeur forceps, ground with a mortar and pestle, and digested at 37°C for 2 hours in DMEM containing 2 mg/mL collagenase type I and 2.5 µg/mL DNase I (Fig 1A). Multiparameter flow cytometry and Uniform Manifold Approximation and Projection (UMAP) a[1,2]nalysis identified distinct subclusters. Functional properties were assessed by colony-forming unit fibroblast (CFU-F) assays, cumulative population doubling analyses, and trilineage differentiation assays (osteogenic, adipogenic, and chondrogenic) (n = 6). Bulk RNA sequencing was performed for each cluster, followed by differential expression analyses to identify age-associated transcriptional changes (n = 6).

RESULTS: UMAP analysis identified six distinct subclusters within the $CD45^{-}CD271^{+}$ population (Fig 1B, C). Cluster1 exhibited the highest CFU-F frequency (Fig 2B), long-term proliferative capacity (Fig 2C), and robust tri-lineage differentiation potential (Fig 2D, E), consistent with a "long-term MSC (LT-MSC)" phenotype. While Cluster2 and Cluster4 retained adipogenic potential, Cluster3 completely lost adipogenic capacity (Fig 2E). Transcriptomic profiling revealed that Cluster1 was enriched for stemness-associated transcription factors (*KLF4, MYC*) and displayed reduced expression of cell cycle regulators (*MKI67, TOP2A*), suggesting a quiescent yet self-renewing state (Fig 3A). In contrast, Cluster2 and Cluster4 exhibited higher expression of adipogenic genes (*ADIPOQ, FABP4, LPL*), and Cluster2 also showed pericyte-like features with elevated smooth muscle gene expression. Cluster3 demonstrated increased expression of osteogenic genes (*SP7, RUNX2, IBSP, COL1A1*). Taken together, Cluster 5 displayed limited proliferation and tri-lineage differentiation potential, resembling short-term MSCs, while Clusters2-4 exhibited lineage-biased features (Fig 3B). Age-dependent analyses showed that Cluster1 cells from elderly donors (≥70 years old) had reduced CFU-F efficiency (Fig 3C), impaired cumulative growth (Fig 3D), and decreased adipogenic and chondrogenic differentiation, while osteogenic potential remained relatively preserved (Fig 3E). Transcriptome profiling further revealed upregulation of *TGFB2, GDF15, FABP4*, and *CEBPA*, along with downregulation of *CTNNB1* (Fig 3F), indicating enhanced TGF-β signaling, reduced Wnt/β-catenin signaling, and an adipogenic-biased state during aging (Fig 3G). Notably, despite transcriptional adipogenic priming, in vitro adipogenic differentiation was paradoxically reduced in aged group, likely reflecting proliferative exhaustion and the absence of in vivo niche cues.

DISCUSSION: In this study, we analyzed the functional and transcriptional heterogeneity of human bone marrow–derived MSCs and identified multiple clusters. Among them, Cluster1, characterized by CD34 expression, exhibited long-term proliferative capacity, self-renewal ability, and tri-lineage differentiation potential, suggesting that this population corresponds to bona fide "LT-MSCs," even though cultured MSCs have traditionally been defined as $CD34^{-}$. Our findings also demonstrate their selective vulnerability to aging, characterized by impaired self-renewal, reduced adipogenic and chondrogenic potential, and rewiring of TGF-β, Wnt, and FGF signaling pathways.

SIGNIFICANCE: This study provides the first functional and transcriptional dissection of human bone marrow MSC subclusters, identifying Cluster1 cells as bona fide LT-MSCs and revealing their selective aging-associated decline. These findings underscore the necessity of targeting distinct MSC subpopulations and signaling pathways to better understand and potentially reverse age-related skeletal degeneration.

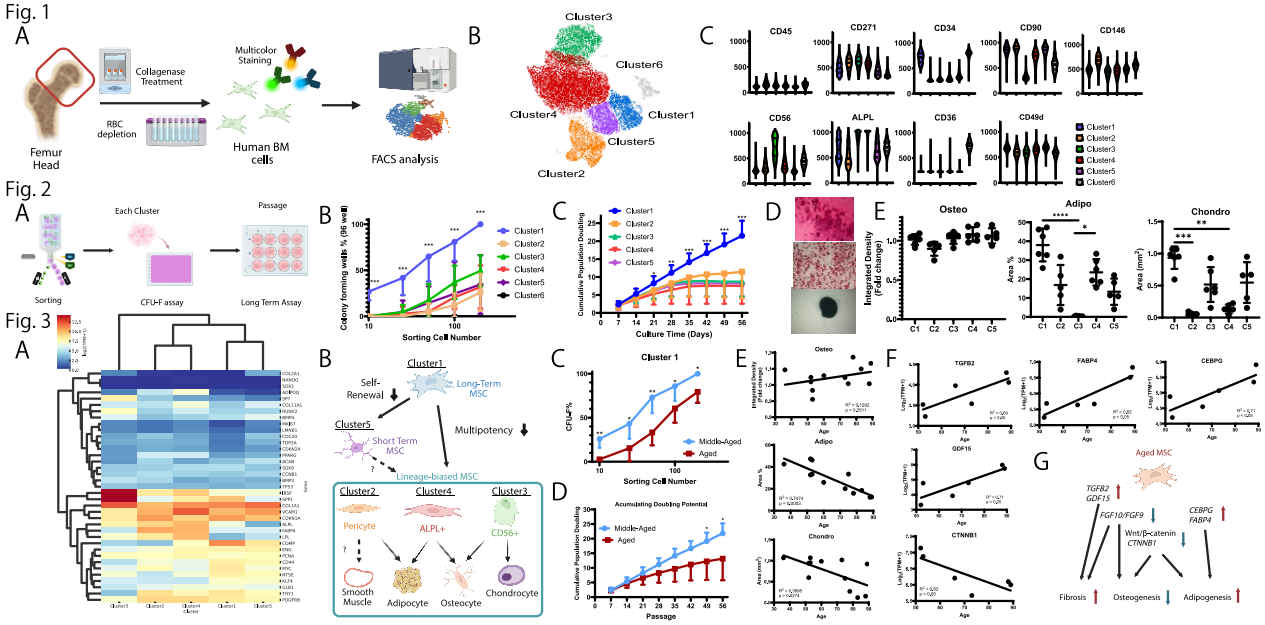

Fig 1. Isolation and clustering of human bone marrow MSCs. A. Workflow for MSC isolation from femoral heads. B. UMAP visualization identifying six distinct $CD45^{-}CD271^{+}$ subclusters. C. Violin plot showing surface marker profiles of each cluster. **Fig 2. Functional characterization of MSC clusters.** A. Workflow for CFU-F/long-term proliferation assays. B/C. CFU-F assay (B) and proliferative capacity (C) of each cluster. D. Representative images showing osteogenic, adipogenic, and chondrogenic differentiation of Cluster1 cells. E. differentiation assays highlighting lineage potential across clusters. **Fig 3. Transcriptomic and age-dependent alterations of MSC clusters.** A. Heatmap of bulk RNA-seq data showing distinct transcriptional profiles. B. Schematic summary of cluster identities. C–E. Age-dependent decline of CFU-F efficiency (C), cumulative growth (D), and differentiation capacity (E) in Cluster1. F. Age-associated gene expression changes. G. Proposed model of signaling rewiring in aged MSCs. Data are presented as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.