

Study on the *in vitro* changes of human bone marrow-related mesenchymal stem cells

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Abstract. Bone marrow mesenchymal stem cells (MSCs) serve a pivotal role in the hematopoietic niche. The present study collected bone marrow samples from individuals across various age groups to investigate the biological characteristics of MSCs. By modifying the bone marrow microenvironment through co-culture techniques, changes in the stemness of MSCs were examined. An *in vitro* hematopoietic co-culture system was established to simulate the impact of MSCs on hematopoietic stem cells. The results demonstrated that the mode of cell-to-cell contact among stem cells is more influential in shaping bone marrow function compared with the effects of aging on these stem cells. Transcriptomic analysis revealed that MSCs serve as essential mediators, with their growth variations being both a consequence and a cause of changes in the bone marrow microenvironment. Furthermore, the decline in hematopoietic function observed in the elderly is a manifestation of this phenomenon. Data from the present study suggest that targeting MSCs is essential for enhancing bone marrow function and improving the outcomes of bone marrow transplantation.

Introduction

Aging is closely associated with the occurrence and progression of senile diseases (1). Previous studies have shown that

stem cell senescence is one of the key factors for the aging and functional decline of tissues and organs, which is also a key target for the prevention and treatment of senile diseases (2,3). With the acceleration of global population aging and the increase in life expectancy, research on the biology of aging, prevention and treatment of aging-related diseases is correspondingly increasing in importance (4,5). The concept of stem cell aging is among the most recent and significant concepts in the study of human aging. Stem cells should not be considered 'immortal', but they rather undergo gradual aging as the body ages (6). This aging process in stem cells results in a progressive decline in their self-renewal and multipotent differentiation capacities, which may lead to uncontrolled proliferation and differentiation. Such changes are key initiating factors for the deterioration of structural integrity, functional decline and the emergence of irreparable damage, subsequently contributing to the onset and progression of related age-related diseases (7-9). Additionally, the decline in bone marrow function is another contributing factor to the development of age-related hematopoietic disorders (10,11).

Mesenchymal stem cells (MSCs) are attractive targets in the emerging field of stem cell aging research (12,13). Due to their low immunogenicity and unique biological properties, bone marrow MSCs have been widely utilized in clinical applications, including hematopoietic stem cell transplantation, tissue repair, treatment of autoimmune diseases and as vectors for gene therapy (14,15). The relationship between the bone marrow hematopoietic microenvironment and hematopoietic stem cells (HSCs) has been frequently likened to a 'soil and seed' analogy (16,17). Bone marrow stromal cells and their precursors, bone marrow MSCs, are essential components of the hematopoietic microenvironment, interacting with hematopoietic progenitor cells that rely on this environment for proliferation, where a dynamic balance is maintained for hematopoiesis (18-20). MSCs serve an important regulatory role in the self-renewal and multidirectional differentiation of HSCs (21,22). Clinical studies have previously shown that reduced or abnormal hematopoietic function is relatively common in the elderly population, presenting as age-related anemia, leukemia and immune deficiencies (23-25). However, the mechanism underlying age-related hematopoietic

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dysfunction remain unclear, warranting further investigation into whether the aging of bone marrow MSCs is associated with this phenomenon.

Age-gradient-associated differences in MSCs are hypothesized to be key to analyzing hematopoietic decline in the elderly. Previous studies on the dynamics of bone marrow aging have identified significant differences between the hematopoietic microenvironments of the elderly and the younger population (26,27). Previous studies have demonstrated that aging of bone marrow hematopoiesis is closely associated with a decrease in IGF1 in the bone marrow microenvironment, and that *Kitl* and *Igf1* expression are coregulated and variable between individual mice at the middle age and expression of these factors is predictive of HSC activation and lymphoid commitment (11,20). To further elucidate these differences and the microenvironmental theory underlying the decline in hematopoietic function in older individuals, the potential impact of the bone marrow microenvironment on hematopoietic function was investigated in the present study by examining the *in vitro* changes in bone marrow MSCs. Additionally, the present study seeks to establish a theoretical foundation for understanding the aging hematopoietic microenvironment, emphasizing that the fate of bone marrow hematopoietic cells is influenced not by intrinsic properties but by their external surroundings. This approach also offers novel therapeutic insights for the prevention and treatment of hematopoietic dysfunction in the elderly.

Materials and methods

Sample collection and categorization. Bone marrow samples were originated from the First Hospital of Chongqing Medical University (Chongqing, China; collection lasted from 1/1/2024 to 1/6/2024). Volunteers (of either sex) without hematologic malignant diseases who had only symptoms of iron-deficiency anemia (IDA) were eligible for collection. The bone marrow samples were then categorized by age, designating volunteers aged ≤ 25 years as the young group (Y group), those aged 45-55 years as the middle-aged group (M group) and those aged ≥ 65 years as the elderly group (O group). Sex and age distribution of patients is included in Table I. The present study was approved (approval no. 2023092) by The Ethics Committee of Chongqing Medical University (Chongqing, China). Written informed consent was obtained from all participants prior to publication of the present study.

Bone marrow puncture. The posterior superior iliac spine was selected to be the site for bone marrow puncture. Specifically, 0.2 ml bone marrow fluid was extracted for the preparation of bone marrow smears, followed by Wright's staining (cat. no. S0217; BIOS) to observe the morphological characteristics of bone marrow cells. A total of 3 ml bone marrow was collected from each volunteer for bone marrow culture.

Bone marrow cytological examination. Qualified bone marrow smears were prepared using Wright's staining technique. In total, 0.5-0.8 ml Wright's Giemsa A solution was added to the smear, ensuring that the staining solution covers the entire specimen for 1 min. Subsequently, Wright's Giemsa B solution was added to the A solution, with a volume that is 2-3X

that of the A solution to facilitate thorough mixing, followed by a staining period of 3-10 min. After washing with water and allowing to dry, the proportions and counts of various hematopoietic cells in the bone marrow were observed under a light microscope (Olympus CKX41; Olympus Corporation) to assess whether the bone marrow sample exhibits signs of hematological disorders or malignant lesions.

ELISA. The supernatant from the bone marrow samples and the co-culture system were isolated before the capture antibody solution was added, which took place in a 96-well plate (cat. no. FCP962; Beyotime Institute of Biotechnology) and was incubated overnight at 4°C. Subsequently, the plate was washed five times with a washing solution to ensure drying. Plasma was then diluted with the sample diluent to concentrations of 1:5, 1:10, 1:50, 1:100 and 1:2,000 to ascertain the optimal concentration. Finally, the concentrations of stem cell factor (SCF; cat. no. QZ-10611), granulocyte macrophage (GM) colony stimulating factor (CSF; cat. no. QZ-10908), IL-3 (cat. no. QZ-10502) and erythropoietin (EPO; cat. no. QZ-10444; all from Quanzhou Jiubang Biotechnology Co., Ltd.) were measured through antigen-antibody interactions, before the absorbance (450 nm) of the cytokines was quantified using a microplate reader (Rayto RT-6100; Rayto Life and Analytical Sciences Co., Ltd.) in conjunction with a standard curve.

***In vitro* culture.** Human bone marrow mononuclear cells (MNCs) were isolated through red blood cell lysis (cat. no. BL503A; Biosharp Life Sciences), lymphocyte separation (cat. no. LTS1077; Tianjin Haoyang Biological Products Technology Co., Ltd.) and density gradient centrifugation (room temperature; 5 min; 16,020 x g). The isolated MNCs were then counted and evenly distributed into 60-mm culture dishes for *in vitro* culture. During the initial stages of cell culture, the medium DMEM F12 (Gibco; Thermo Fisher Scientific, Inc.) supplemented 10% fetal bovine serum (FBS; cat. no. FSP500; Shanghai ExCell Biology, Inc.) was partially replaced every 3 days until adherent-dependent cells emerged. Subsequently, a complete medium exchange and cell passaging procedures were performed to separate and purify the adherent-dependent cells. Furthermore, during the early phases of the *in vitro* culture of bone marrow MNCs, the supernatant cells were collected for additional *in vitro* culture. The supernatant cells were treated with a magnetic bead sorting kits (cat. nos. 130-092-263 and 130-100-453; Miltenyi Biotec GmbH), and these cells were successively treated with CD38- and CD34-labeled magnetic beads, respectively; and CD34-positive and CD38-negative HSCs were collected for co-culture operation under the action of a magnetic field by the negative and positive sorting methods, respectively.

Flow cytometry. After counting (1×10^6), adherent-dependent cells and suspended cells were allocated to Eppendorf tubes separately and treated with 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) blocking buffer under light conditions for 30 min. The adherent cells were stained with specific surface marker antibodies (dilution ratio 1:100) at 4°C for 30 min in the dark, including CD73 (cat. no. 561258), CD90 (cat. no. 555595), CD105 (cat. no. 561443), CD14 (cat. no. 557153), CD34 (cat. no. 555821), CD45 (cat.

Table I. Demographic characteristics of the volunteers.

Variable	Total n=184	Y n=49	M n=51	O n=84	χ^2	P-value
Female, n (%)	96 (52.17)	27 (55.10)	29 (56.86)	40 (47.62)	1.316	0.518
Male, n (%)	88 (47.83)	22 (44.90)	22 (43.14)	44 (52.38)		

Data are presented as number (proportion) and analyzed using chi-square test. Y, young group (10-25 years); M, middle-age group (45-55 years); O, old group (75-90 years).

no. 555482) and human leukocyte antigen (HLA-DR; cat. no. 555811), whilst the suspended cells were stained with the appropriate antibodies against CD34 (cat. no. 555821) and CD38 (cat. no. 567147) (all from BD Biosciences) before identification using flow cytometry. Following washing with PBS (cat. no. G4250-500ML; Wuhan Servicebio Technology Co., Ltd.), the isolated and purified adherent cell pellet was collected, resuspended in 100 μ l PBS and mixed gently with 900 μ l of pre-chilled 75% ethanol to ensure complete homogenization for subsequent cell cycle assessment. After counting, these cells were also used for apoptosis measurement and subsequently resuspended in 500 μ l PBS. Finally, the results of the cell cycle and apoptosis analyses were obtained through flow cytometry (BD FACSAria™ Fusion; BD FACSDiva™ Software; BD Biosciences).

Co-culture. The present study used multiple co-culture systems. The first system utilized bone marrow plasma from groups Y and O to intervene in the proliferation of bone marrow MSCs across the respective groups. The second system leveraged the suspended proliferative characteristics of HSCs and the adherent proliferative characteristics of bone marrow MSCs. In this system, Y group HSCs were selected as the experimental subjects to investigate their hematopoietic activity, with bone marrow MSCs from each group serving as intervention factors to establish an *in vitro* hematopoietic co-culture system. The third system involved co-culturing the bone marrow MSCs from group Y with the differentiated adherent bone marrow cells from group O. Subsequently, the pre-treated hematopoietic cells were collected for *in vitro* hematopoiesis assays and assessment of colony forming unit (CFU)-erythroid (E), burst forming unit (BFU)-E, CFU-GM and CFU-granulocytes/erythroids/macrophages/megakaryocytes (GEMM) formation.

Humanized hematopoietic colony formation assay. Y group hematopoietic stem progenitor cells were collected from the hematopoietic co-culture system, added to MethoCult® medium (cat. no. 04034; STEMCELL Technologies, Inc.), shaken well and then plated into culture dishes for incubation at 37°C with 5% CO₂ for 14 days. The types and counts of colonies were assessed using an inverted microscope (Olympus CKX41; Olympus Corporation). (A single CFU-E is formed by 8-200 erythrocytes; a single BFU-E is formed by >200 erythrocytes; a single CFU-GM is formed by at least 20 granulocytes and macrophages; a single CFU-GEMM is formed by >500 cells containing erythrocytes, granulocytes and macrophages).

EdU incorporation. Following intervention with bone marrow plasma, equal amounts of Y group bone marrow MSCs were seeded into culture dishes to restore normal proliferation. An equal volume of pre-warmed EdU (cat. no. C0078S; Beyotime Institute of Biotechnology) working solution (final concentration of EdU at 10 μ M) and culture medium was then added to the dishes, followed by an additional 5-h incubation at 37°C with 5% CO₂. After EdU labeling was completed, cells were treated with fixation and permeabilization solutions for 15 min, incubated with Click reaction solution in the dark at room temperature for 30 min and then stained for nuclei (cat. no. C1005; Beyotime Institute of Biotechnology). Finally, proliferation was analyzed using fluorescence detection.

Immunofluorescence. After intervention with bone marrow plasma, equal amounts of M group bone marrow MSCs were seeded into culture dishes to restore normal proliferation. The cells were fixed with 4% paraformaldehyde (room temperature; 15 min) and permeabilized with Triton X-100 (room temperature; 15 min). Subsequently, they were incubated with antibodies for senescence-associated proteins p53 (cat. no. BF8013; Affinity Biosciences) (dilution ratio 1:500) and p21 (cat. no. AF6290; Affinity Biosciences) (dilution ratio 1:500) at room temperature for 1 h, followed by blocking (cat. no. P0260; Beyotime Institute of Biotechnology) (room temperature; 10 min) and incubation with secondary antibodies (DyLight 488, Goat Anti-Mouse IgG; cat. no. A23210; and DyLight 549, Goat Anti-Rabbit IgG; cat. no. A23320; both from Abbkine Scientific Co., Ltd.) (dilution ratio 1:100) at room temperature for 1 h in the dark to form antigen-antibody complexes. Fluorescence was then observed and quantified using a confocal microscope (AX/AX R with NSPARC; Nikon Corporation).

Osteogenesis and adipogenesis. Equal amounts of O group bone marrow MSCs, following intervention with bone marrow plasma, were seeded into culture dishes to restore normal proliferation. Upon reaching 70% confluence, the culture medium was removed, before 2 ml OriCell® Human BMMSC Osteogenic Differentiation Medium (cat. no. HUXMA-90021; Cyagen Biosciences, Inc.) was added into each dish, with the medium replaced every 3 days. After 2-4 weeks of induction, alkaline phosphatase staining (cat. no. G1481; Beijing Solarbio Science & Technology Co., Ltd.) was performed to assess osteogenic differentiation. Similarly, when the bone marrow MSCs attained 70% confluence, the upper culture medium was removed and 2 ml OriCell® Human BMMSC Adipogenic Differentiation Medium A (cat. no. HUXMA-90031; Cyagen

Biosciences, Inc.) was added to each dish. Following 3 days of induction, the medium was aspirated and 2 ml OriCell® Human BMMSC Adipogenic Differentiation Medium B (cat. no. HUXMA-90031; Cyagen Biosciences, Inc.) was introduced. After 1 day, medium B was discarded and medium A was reinstated. The two media were thereafter alternated in this manner whilst regularly monitoring cell status. Finally, Oil Red O staining (cat. no. G1262; Beijing Solarbio Science & Technology Co., Ltd.) was used to evaluate the adipogenic differentiation of the cells.

Cell counting kit-8 (CCK-8). Bone marrow MSCs were seeded into 96-well plates, with 100 μ l (containing 2,000 cells) added to each well. Following this, 10 μ l CCK-8 (cat. no. C0038; Beyotime Institute of Biotechnology) solution was added to each well and the cells were incubated in a culture incubator (cat. no. BB150-2TCS-L; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂ for 6 h. Absorbance was measured at 450 nm and cell viability was analyzed based on the results.

Transmission electron microscopy. Bone marrow MSCs were digested with trypsin (cat. no. 25200072; Gibco; Thermo Fisher Scientific, Inc.), counted and the cell pellet ($\geq 10^6$) was collected. Next, 3% paraformaldehyde fixative was gently added along the wall of the tube for at least 2 h at room temperature. Cell precipitates were stained (room temperature; 3 h) with heavy metal salts and embedded (room temperature; 1 h) in epoxy resin. After making ultrathin sections, (70–100 nm) they were used for observation. The prepared samples were subsequently sent to the Electron Microscopy Laboratory of Chongqing Medical University for observation.

β -galactosidase staining. Bone marrow MSCs were cultured to 70% confluency and then the upper layer of medium was removed. Following a wash with PBS, β -galactosidase fixation solution was added and the cells were fixed at room temperature for 15 min. After fixation, PBS was used to wash away the fixative and the cells were incubated overnight at 37°C with β -galactosidase staining solution (cat. no. C0602; Beyotime Institute of Biotechnology). For suspended hematopoietic stem progenitor cells, these cells were collected by centrifugation and stained using the same method. The stained cells were then placed on a glass slide for observation under an optical microscope.

Transcriptome sequencing (RNA-Seq). The isolated and purified Y group and O group bone marrow MSCs were sent to the Beijing Genomics Institute for RNA-Seq (BGI Genomics Co., Ltd.). The sequences and expression information of the transcripts from both groups were analyzed (<https://biosys.bgi.com/#/report/login>) using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment methods. The sequencing results were processed using the STRING database (<https://cn.string-db.org/>) and Cytoscape software (Cytoscape 3.7.2; <https://cytoscape.org/download.html>) to construct a protein-protein interaction (PPI) analysis of differentially expressed genes.

Reverse transcription-quantitative PCR (RT-qPCR). Under enzyme-free conditions, RNA was extracted from human bone

marrow MSCs using an RNA extraction kit (cat. no. R0018M; Beyotime Institute of Biotechnology). Primer sequences for the genes to be validated are included in Table SI. Reverse transcription was performed using RT kit (cat. no. RR037A; Takara Bio, Inc.) according to the manufacturer's protocol, followed by qPCR using SYBR Green qPCR Master Mix (cat. no. 639676; Takara Bio, Inc.) on the circulator real-time detection system (Bio-Rad). Actin was used as the reference gene. (Thermal cycle conditions: the first step of pre-denaturation 95°C for 30 sec, the second step 95°C for 10 sec and 60°C for 30 sec, cycle 40 times). The quantification method was the $\Delta\Delta C_q$ method (28).

Statistical analysis. All experiments were performed in triplicate at a minimum. Statistical analysis was conducted using GraphPad Prism 9.5 (Dotmatics). Measurement data were expressed as the mean \pm SD. Comparisons between two groups of data that met the criteria of normal distribution and homogeneity of variance were performed using an unpaired t-test. For comparisons among multiple groups, one-way analysis of variance was employed, followed by Tukey's post hoc test. For comparisons of data between groups at different time points, repeated measures ANOVA was utilized, with Bonferroni correction for post hoc analysis. Pearson correlation analysis was used to assess the relationships among various indicators. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Analysis of age-related differences in bone marrow function. For the present study examining the causes of diminished bone marrow function in the elderly, 184 anemia samples were collected using random sampling methodology (Table SII). Bone marrow smears indicated that the proportions of myeloid, erythroid and lymphoid lineages, in addition to the counts of megakaryocytes, were normal across all samples, suggesting the absence of hematological malignancies (Fig. 1A–C). Statistical analysis revealed that the O group comprised the highest proportion of samples, suggesting a significant degradation of hematopoietic function in the elderly. Furthermore, there were no sex differences among the groups, indicating that the decline in hematopoietic function did not vary between elderly male and female population (Table I).

The lipid composition in bone marrow samples exhibited differential levels based on age, with the O group showing the highest lipid content (Fig. 1D and F). MNCs were obtained from the bone marrow samples using density gradient centrifugation, where the data indicated a gradual decline in the number of MNCs as age increased (Fig. 1E and G). Assessments of hematopoietic-related factors in bone marrow plasma showed reduced levels of SCF, GM-CSF, IL-3 and EPO in the O group (Fig. 1H). *In vitro* cultured, magnetically-sorted bone marrow hematopoietic stem progenitor cells (Fig. 1I and J) were subsequently analyzed for the expression of surface markers using flow cytometry, revealing that CD34 was positive and CD38 was negative, consistent with the definition of bone marrow HSCs (Fig. 2A). Statistical analysis indicated that the content of HSCs sourced from the O group was the lowest, whilst that from the Y group was the highest (Fig. 1K). In the early stages

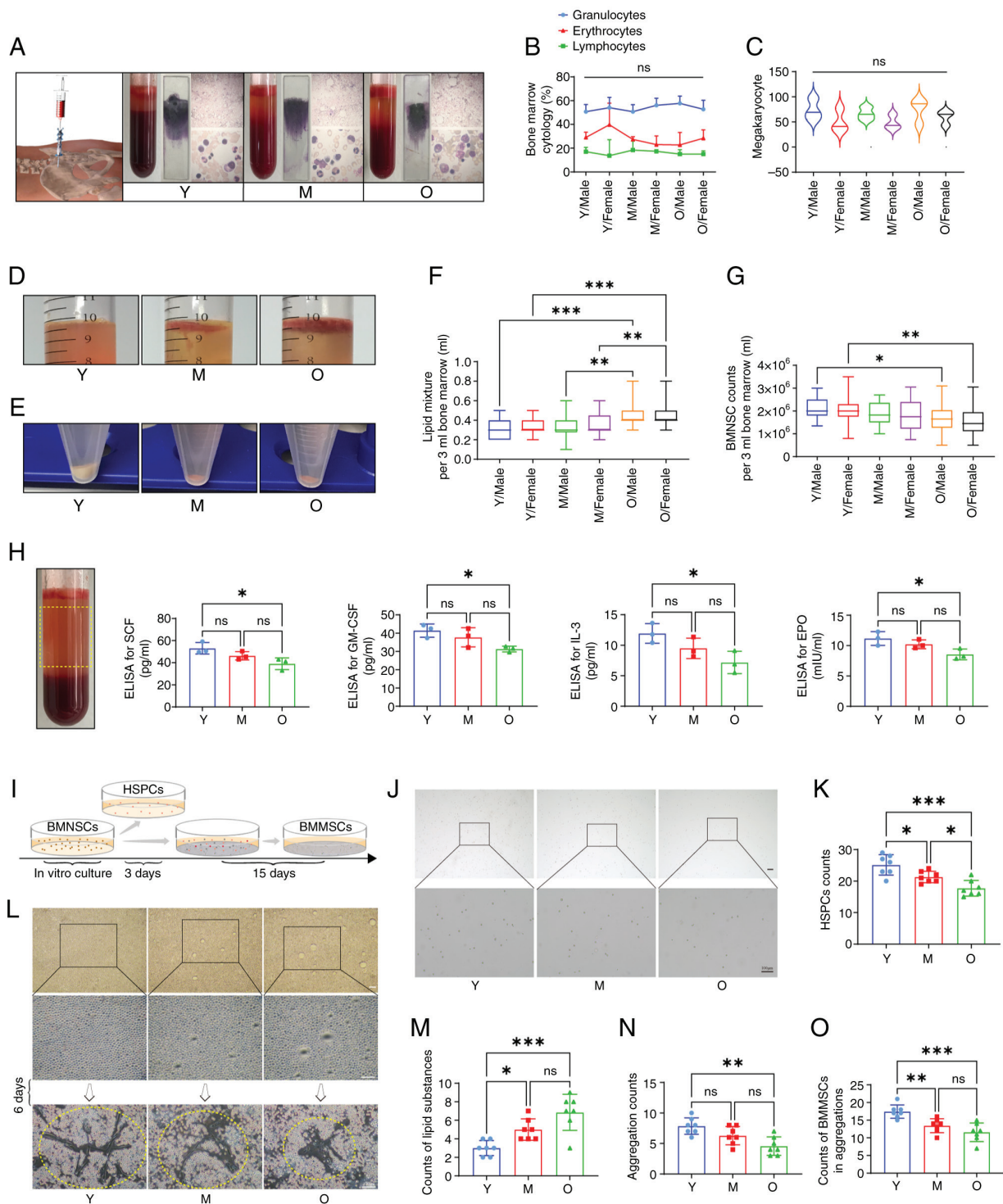


Figure 1. Analysis of age-related differences in bone marrow associated with senile anemia. (A) Collection of bone marrow samples from the posterior superior iliac spine. (B) Clinical examination of bone marrow cytology for the samples (n=184). (C) Assessment of megakaryocyte lineage in bone marrow samples (n=184). (D) Variations in marrow adiposity. (E) Differences in the extraction methods for MNCs from the bone marrow. (F) Statistical analysis of lipid mixture content in each bone marrow sample (n=184). (G) Statistical analysis of the number of MNCs extracted from each bone marrow sample (n=184). (H) Examination of hematopoietic-related factors SCF, GM-CSF, IL-3 and EPO in the bone marrow (n=3). (I) Schematic representation of bone marrow cell culture. (J) *In vitro* culture and observation of hematopoietic stem progenitor cells following magnetic bead sorting. (K) Statistical analysis of the number of hematopoietic stem progenitor cells across various age groups (n=7). (L) *In vitro* culture and early proliferation observation of MNCs from bone marrow across different age groups, alongside bone marrow MSCs. (M) Statistical analysis of the production of suspended lipids during the *in vitro* culture of MNCs from bone marrow (n=7). (N) Statistical analysis of aggregation regions during the early adherent growth phase of bone marrow MSCs (n=7). (O) Statistical analysis of variations in the number of bone marrow MSCs in the early aggregation regions (n=7). *P<0.05, **P<0.01 and ***P<0.001. MNCs, mononuclear cells; SCF, stem cell factor; GM-CSF, granulocyte colony stimulating factor; EPO, erythropoietin; MSCs, mesenchymal stem cells; ns, no significance.

of the *in vitro* culture of bone marrow MNCs (Fig. 1L), the upper culture medium of the O group exhibited the highest

lipid production (Fig. 1M), although this phenomenon was not observed in subsequent culture and passing stages. On

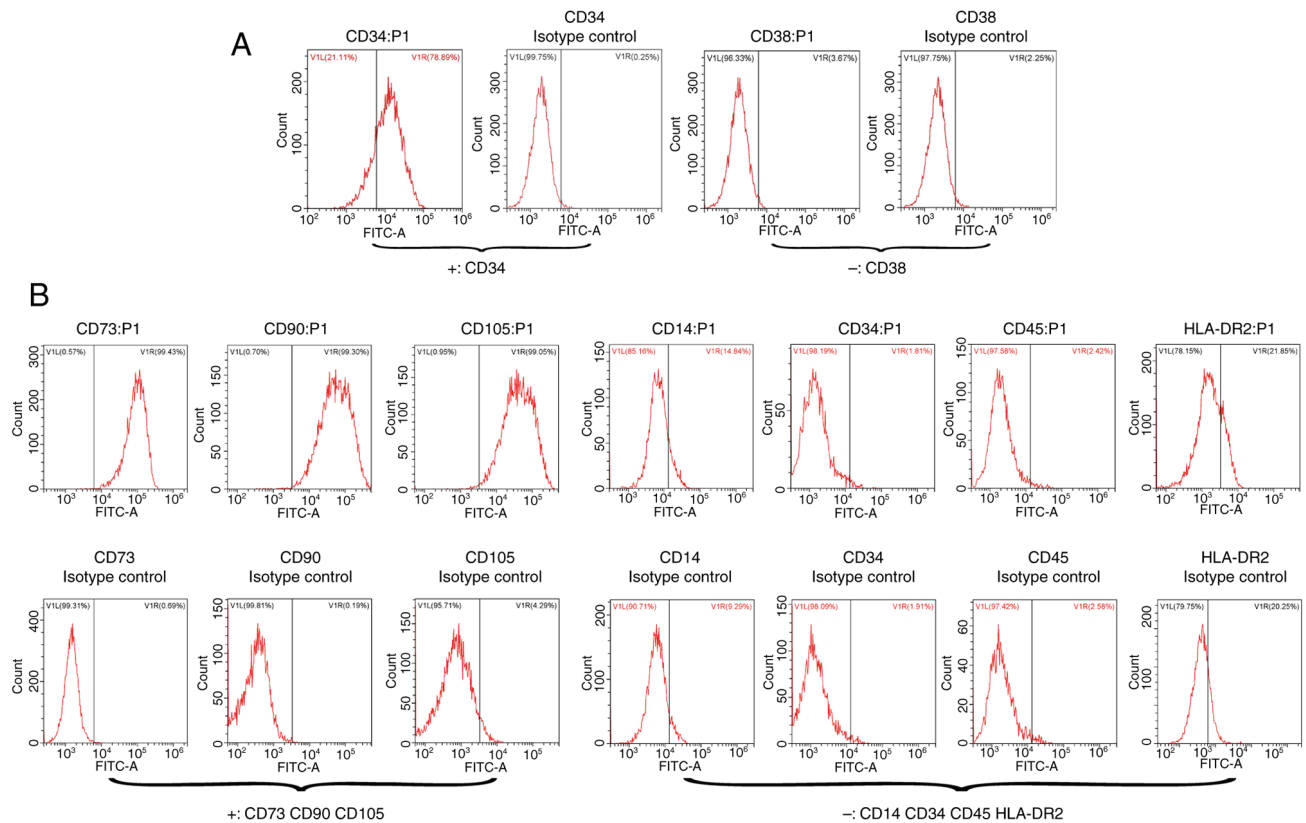


Figure 2. *In vitro* identification of bone marrow cells. (A) Identification of hematopoietic stem progenitor cells (CD34⁺ CD38⁻). (B) Identification of bone marrow mesenchymal stem cells (+: CD73, CD90 and CD105; -: CD14, CD34, CD45, HLA-DR2). HLA, human leukocyte antigen.

day 6 of *in vitro* culture, bone marrow MSCs displayed trends of adherent aggregative proliferation under microscopy. Flow cytometry for the expression of surface markers demonstrated that CD73, CD90 and CD105 were positive, whilst CD14, CD34, CD45 and HLA-DR2 were negative, aligning with the definition of bone marrow MSCs (Fig. 2B). Compared with the other groups, the O group exhibited the smallest clustered area of bone marrow MSCs and the fewest number of cells forming clusters (Fig. 1N and O). These results suggested that the decline in bone marrow function among the elderly population is attributed to a reduction in the number of stem cells and a deterioration of their functional capacity, which is closely associated with the bone marrow microenvironment. However, the causal relationship remains to be validated.

Changes in the bone marrow microenvironment lead to a decrease in MSC numbers and function. To further investigate the effects of alterations in the bone marrow microenvironment on bone marrow MSCs, the *in vitro* proliferation of MSCs derived from bone marrow plasma samples of the Y and O groups across age gradients was next assessed (Fig. 3A). Control group results (no intervention) exhibited distinctively dense contact phenomena in MSCs during extensive proliferation (Fig. 3B). Microscopic observations revealed that MSCs in the experimental Y group exhibited a regular and organized growth pattern, forming membrane ridges at points of contact (Fig. 3C). Under identical culture conditions, MSCs in the experimental O group displayed fused growth characteristics, with unclear cell boundaries and varied morphologies (Fig. 3D). EdU assays demonstrated a reduced proliferative

capacity in Y group MSCs in the O group compared with that in the control group (Fig. 3E and F). Immunofluorescence results indicated that Y group bone marrow plasma could decrease the expression of senescence-associated proteins p53 and p21 in M group MSCs, whereas M group MSCs exhibited increased levels of p53 and p21 following intervention with O group bone marrow plasma (Fig. 3G and H). Regarding the multidirectional differentiation capacity of bone marrow MSCs, a complementary trend toward osteogenic and adipogenic differentiation was observed (29). Differentiation results suggested that, compared with those in the control group, Y group bone marrow plasma enhanced the osteogenic potential of O group MSCs whilst diminishing their adipogenic capacity (Fig. 3I and J). During the *in vitro* culture phase, Y group bone marrow plasma significantly increased the viability of MSCs across all groups, whilst O group plasma led to decreased cell viability in all groups (Fig. 3K). Electron microscopy results revealed that MSCs from the experimental Y group contained abundant intracellular materials and active organelles, most notably the endoplasmic reticulum. By contrast, MSCs from the experimental O group exhibited irregular membrane folds, enlarged cell bodies, increased translucent intracellular materials and significant cell differentiation (Fig. 3L). These findings suggested that bone marrow MSCs are influenced by their growth environment, where modulating such bone marrow microenvironment will likely effectively alter the stemness of these cells (30,31).

Changes in MSCs lead to a decrease in hematopoietic capacity of HSCs. To investigate the direct relationship

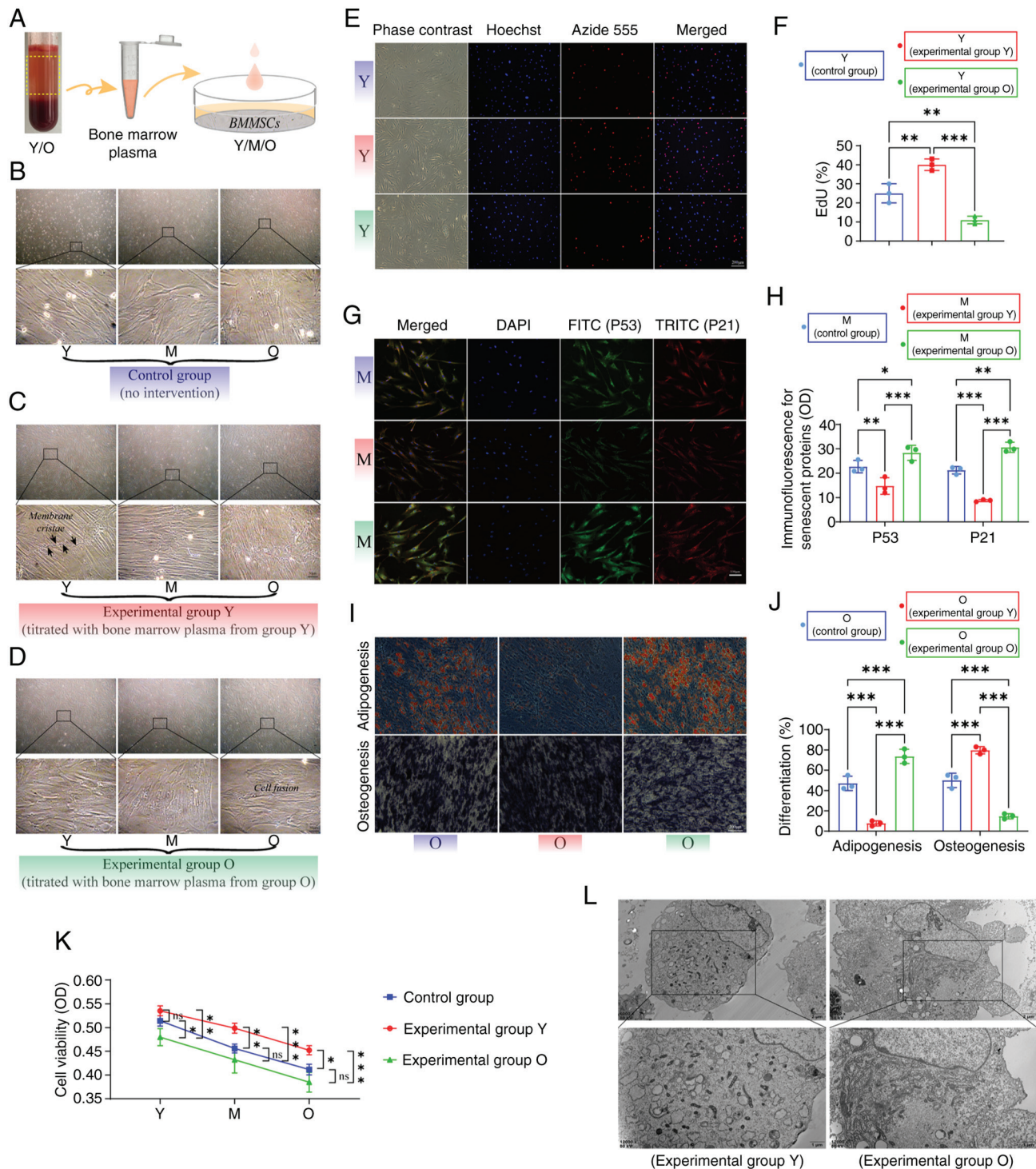


Figure 3. Effects of bone marrow plasma on the stemness of bone marrow MSCs. (A) Schematic representation of the intervention of bone marrow plasma in the *in vitro* culture of bone marrow MSCs. (B) Growth conditions of isolated and purified bone marrow MSCs *in vitro*. (C) Growth status of bone marrow MSCs in each group following intervention with Y group bone marrow plasma. (D) Growth status of bone marrow MSCs in each group following intervention with O group bone marrow plasma. (E) Assessment of the proliferation capacity of Y group bone marrow MSCs after exposure to various bone marrow plasma interventions. (F) Statistical analysis of EdU assay results (n=3). (G) Expression of senescence-associated proteins p53 and p21 in M group bone marrow MSCs following different bone marrow plasma interventions. (H) Statistical analysis of immunofluorescence results for senescence-associated proteins (n=3). (I) Changes in the osteogenic and adipogenic potential of O group bone marrow MSCs after different bone marrow plasma interventions. (J) Statistical results of osteogenic and adipogenic differentiation (n=3). (K) Cell viability of bone marrow MSCs in each group under varying influences of bone marrow plasma (n=3). (L) Electron microscopy observations of bone marrow MSCs following intervention with Y group and O group bone marrow plasma, highlighting notable changes in the endoplasmic reticulum and plasma membrane. *P<0.05, **P<0.01 and ***P<0.001. MSCs, mesenchymal stem cells.

between age-related changes in bone marrow stem cells and hematopoiesis in the elderly population, bone marrow MSCs from various age groups were co-cultured with HSCs from

the Y group using *in vitro* modeling (Fig. 4A). The MSCs co-cultured for this experiment exhibited robust proliferative capabilities *in vitro* (Fig. 4B). Statistical analysis indicated that

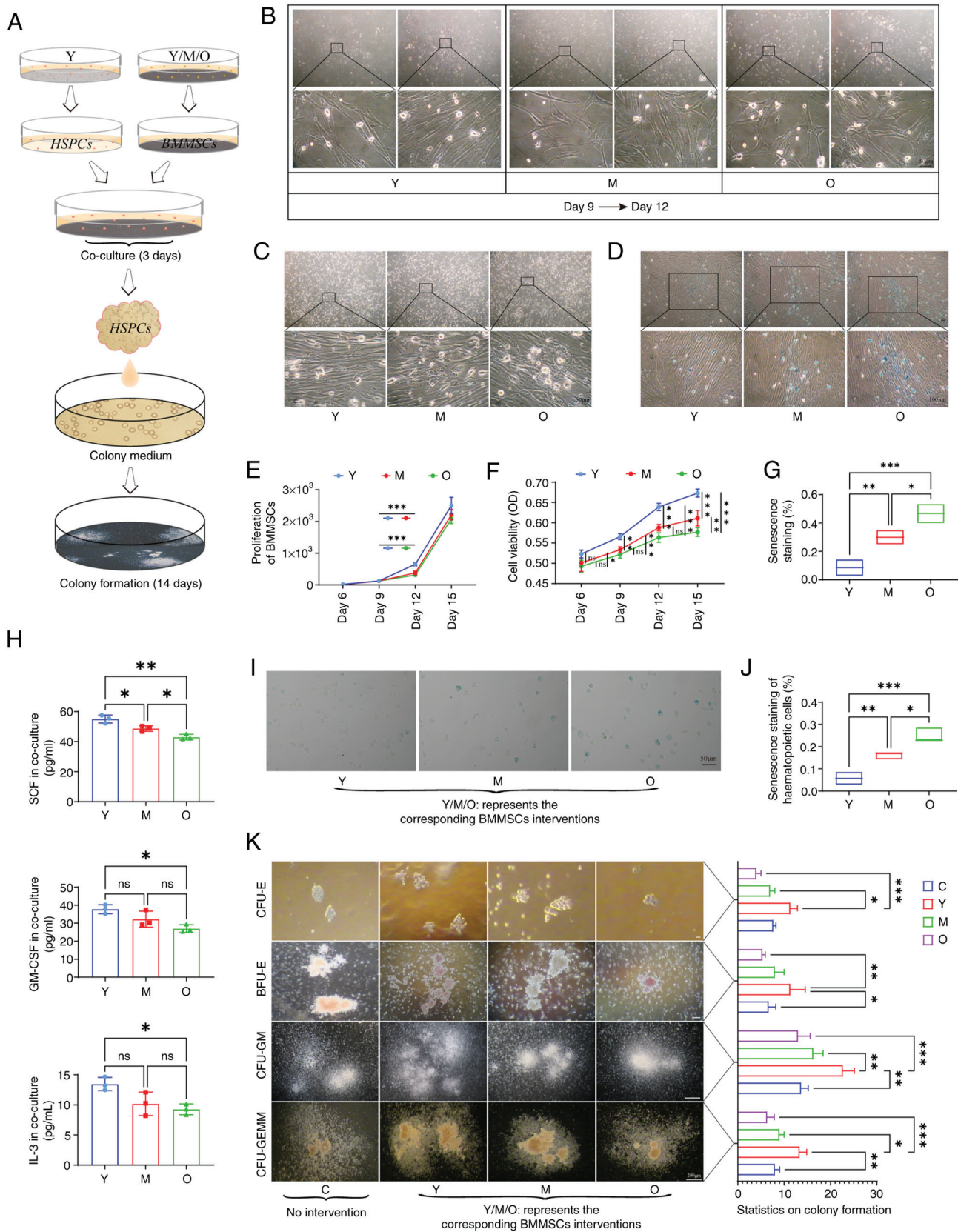


Figure 4. Differential manifestations of aging hematopoiesis *in vitro*. (A) Schematic representation of the co-culture of hematopoietic stem progenitor cells from the Y group with MSCs from the Y/M/O groups. (B) Early-stage proliferation of bone marrow MSCs *in vitro*. (C) Observation of cell fusion phenomena during the *in vitro* culture of bone marrow MSCs. (D) β -Galactosidase staining reaction indicating the senescence of bone marrow MSCs during *in vitro* proliferation. (E) Statistical analysis of the *in vitro* proliferation of primary cultured bone marrow MSCs (n=7). (F) Assessment of cell viability in bone marrow MSCs at various stages of primary culture (n=3). (G) Statistical analysis of senescence-positive staining (n=3). (H) Examination of hematopoietic-related factors SCF, GM-CSF and IL-3 in the co-culture supernatant (n=3). (I) Assessment of senescence in Y group hematopoietic stem progenitor cells post co-culture. (J) Statistical analysis of senescence-positive staining in Y group hematopoietic stem progenitor cells following co-culture (n=3). (K) Differential analysis and statistical evaluation of hematopoietic colony formation by Y group hematopoietic stem progenitor cells after co-culture (n=3). *P<0.05, **P<0.01 and ***P<0.001. MSCs, mesenchymal stem cells; SCF, stem cell factor; GM-CSF, granulocyte macrophage colony stimulation factor.

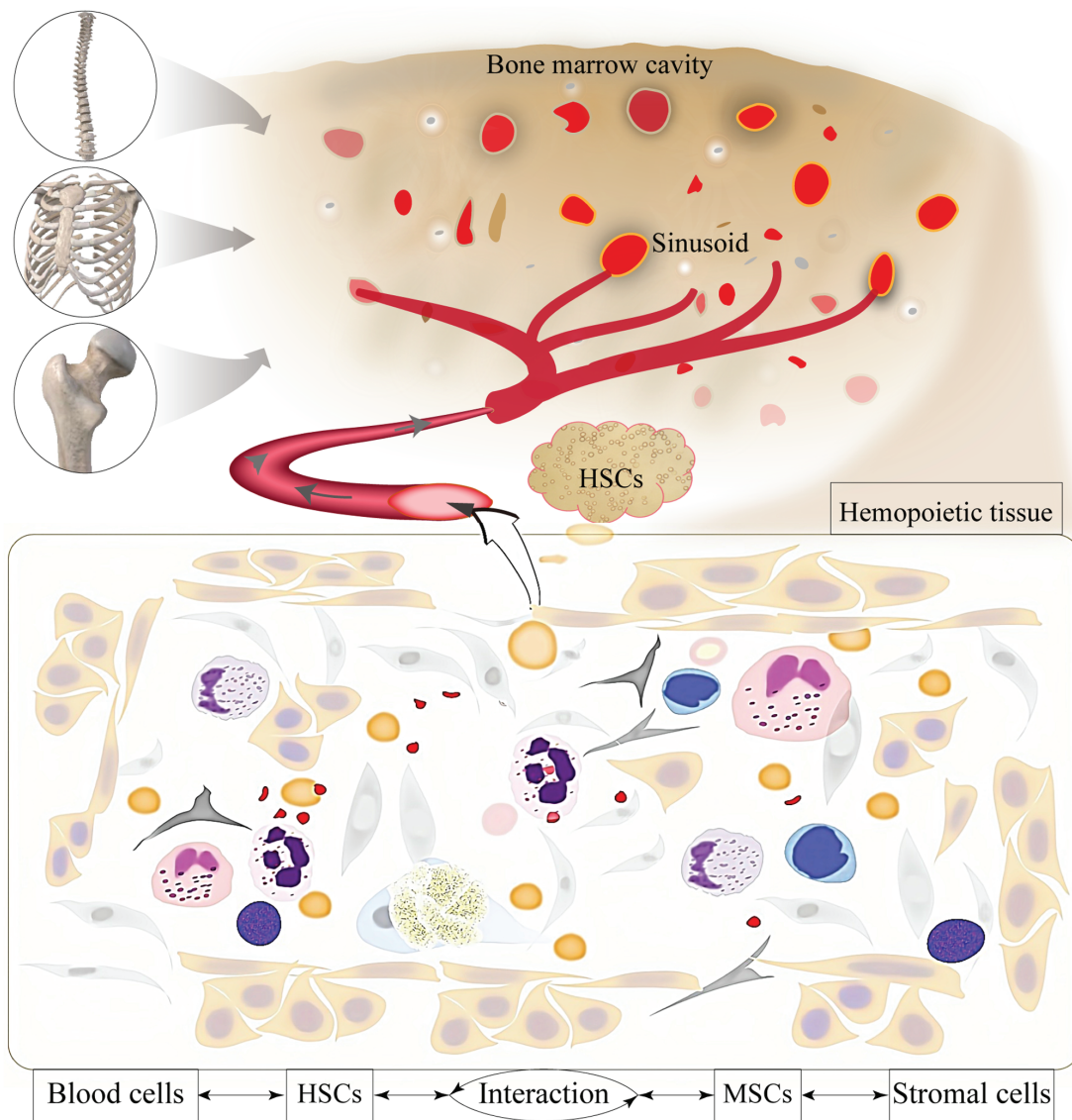


Figure 5. Close relationship between bone marrow MSCs and bone marrow hematopoietic stem progenitor cells. 'Soils' and 'seeds'. Schematic diagram of the human bone marrow hematopoietic induction microenvironment. The bone marrow microenvironment in which hematopoietic cells live and function is depicted in this image with important bone marrow stromal cells as representatives. The bone marrow microenvironment is subject to a range of alterations during the life activities of the organism, such that the composition of the microenvironment changes with age, which in turn affect the function of hematopoietic cells. The stemness of hematopoietic stem progenitor cells may diminish with age, but they are primarily influenced by the surrounding environment to meet the hematopoietic needs of the organism. Therefore, fate may not be dependent on the hematopoietic cells themselves, but rather in the environment surrounding them. MSCs, mesenchymal stem cells.

during the early stages of proliferation *in vitro*, Y group MSCs displayed the highest proliferation capacity, whilst there was no statistically significant difference in cell numbers among the groups as culture time extended (Fig. 4E). Cell viability assessments revealed that primary cultured MSCs maintained robust cell viability at all stages (Fig. 4F). The proliferation of stem cells is closely associated with cell contact, where prolonged *in vitro* culture time may reduce the differences in stemness among the stem cells (32-35). Microscopic observations indicated that MSCs predominantly exhibited a spindle shape, where after extensive proliferation, a fusion growth phenomenon was observed, particularly pronounced in the O group (Fig. 4C). β -galactosidase staining for cellular aging indicated a significant presence of senescence-positive MSCs at areas of severe cell fusion growth (Fig. 4D and G). The levels of hematopoietic-related factors in different co-culture

system supernatants were next measured and it was found that O group MSCs, as intervention factors, exhibited the lowest expression of SCF, GM-CSF and IL-3 when co-cultured with Y group HSCs (Fig. 4H). Compared with that in other systems, co-culturing with O group MSCs resulted in the significant aging of Y group HSCs (Fig. 4I), as evidenced by the increase in the proportion of senescence-positive HSCs (Fig. 4J). In human bone marrow colony formation assays, CFU-E and BFU-E colonies formed earliest, where statistical analysis of colony numbers indicated age-related differences among the experimental groups, with the O group exhibiting the poorest colony formation capability. In terms of CFU-GM and CFU-GEMM formation, the number of colonies and the density of cells within colonies differed significantly. Y group HSCs co-cultured with Y group MSCs formed a greater number of colonies with higher cell density, whilst Y group

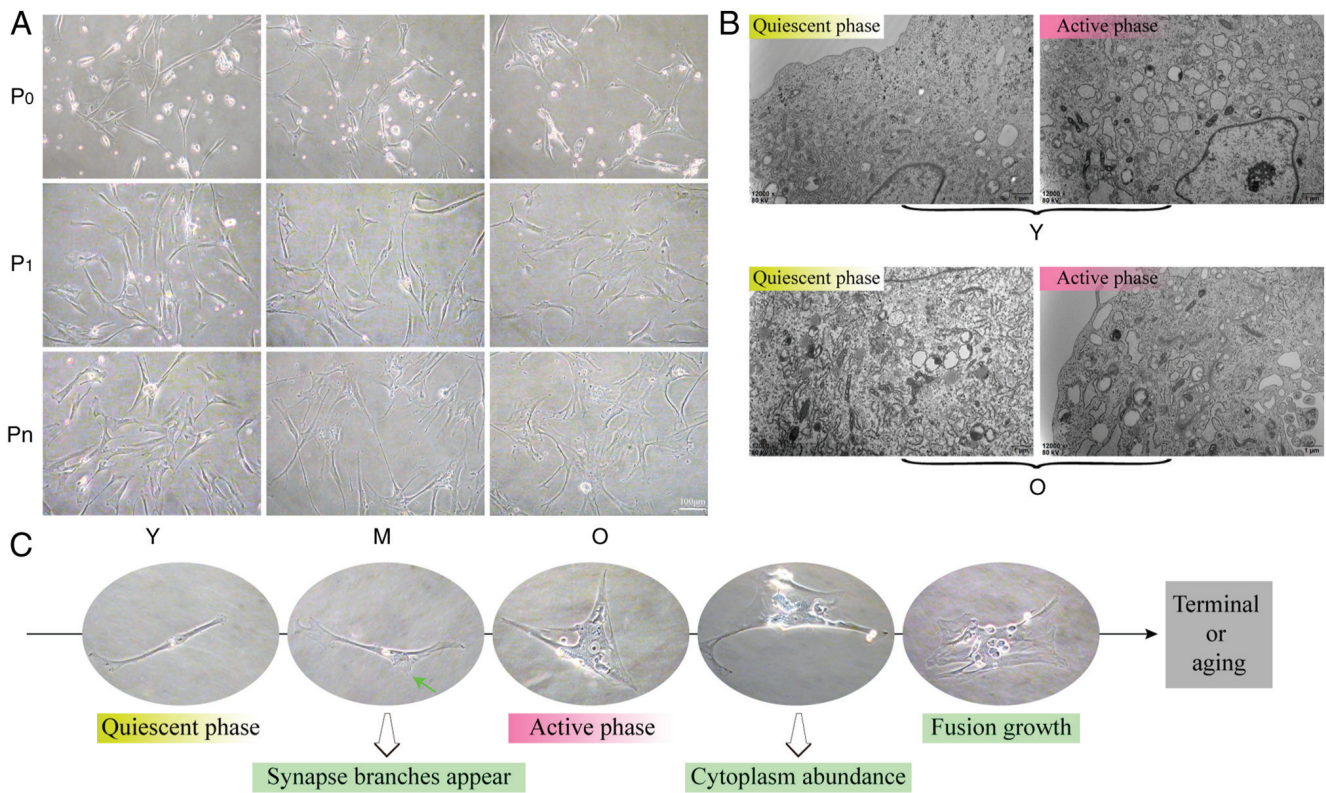


Figure 6. *In vitro* growth morphology of MSCs. (A) Morphological features of bone marrow MSCs at various stages of *in vitro* culture. (B) Active and quiescent states of Y group and O group bone marrow MSCs as observed under electron microscopy. (C) Functional and morphological evolution of bone marrow MSCs. MSCs, mesenchymal stem cells.

HSCs co-cultured with O group MSCs produced the fewest colonies with the lowest cell density (Fig. 4K). These results suggested that the state of MSCs coexisting with HSCs in the bone marrow will likely affect the hematopoietic capacity of HSCs (Fig. 5).

Loss of stemness in MSCs during *in vitro* culture. Bone marrow MSCs serve an important role in bone marrow function. To investigate the growth characteristics of MSCs, their morphological features were analyzed at various stages of *in vitro* culture. During the early primary culture of Y group MSCs, cells exhibited a spindle shape, characterized by relatively large nucleoplasm and a small number of protruding branches at the ends. As age increased or as the *in vitro* culture progressed, the intracellular content of MSCs became more abundant, where the nucleoplasm decreased and the spindle characteristics gradually diminished, resulting in a transformation to polygonal or irregular shapes until differentiation into terminal cells. This morphology was commonly observed in the long-term *in vitro* culture of O group MSCs (Fig. 6A and C). Culturing results indicated that the various morphologies of MSCs coexisted in the different age groups, each with specific proportions and patterns. Electron microscopy observations revealed that MSCs primarily existed in the following two states: A quiescent phase and an active phase. Compared with quiescent MSCs, those in the active phase exhibited increased organelle function, particularly in the endoplasmic reticulum, along with an increase in vesicular structures (Fig. 6B). Cell cycle analysis showed that MSCs predominantly remained in the G₁ phase during *in vitro* culture,

with age-related differences based on the culture stage. In the primary culture (P0 stage) of Y group MSCs, the proportion of cells in the G₁ phase was the highest, whilst in the passaged culture (Pn, n≥3) of O group MSCs, the proportion of cells in the G₁ phase was the lowest (Fig. 7A and C). Apoptosis results indicated that as age increased and culture time extended, the proportion of apoptotic MSCs was also increased, with a particularly rapid rise in late-stage apoptosis (Fig. 7B and D). These findings suggested that the stemness of MSCs evolves in relation to differences in origin and culture stage, highlighting the importance of the *in vitro* culture phase as a critical factor for intervening in their stemness.

Identifying key causes of MSC stemness loss through transcriptomics analysis. In the investigation of the key factors influencing the differences in stemness among bone marrow MSCs, early in the primary culture of the O group, certain bone marrow materials were found to exhibit substratum adherence, resembling the behavior of MSCs, primarily consisting of morphologically diverse bone marrow stromal cells, alongside the generation of highly refractive bone marrow terminal substances. Under these conditions, MSCs demonstrated reduced proliferation (Fig. 8A and B). Passage of these bone marrow materials showed no significant changes (Fig. 8C and D). The MSCs were then isolated from these materials for passage culture, where the results indicated that MSCs could effectively proliferate when the influence of the bone marrow materials was removed (Fig. 8E). When the efficiently proliferating Y group MSCs were co-cultured with these bone marrow materials, the morphology of the Y group

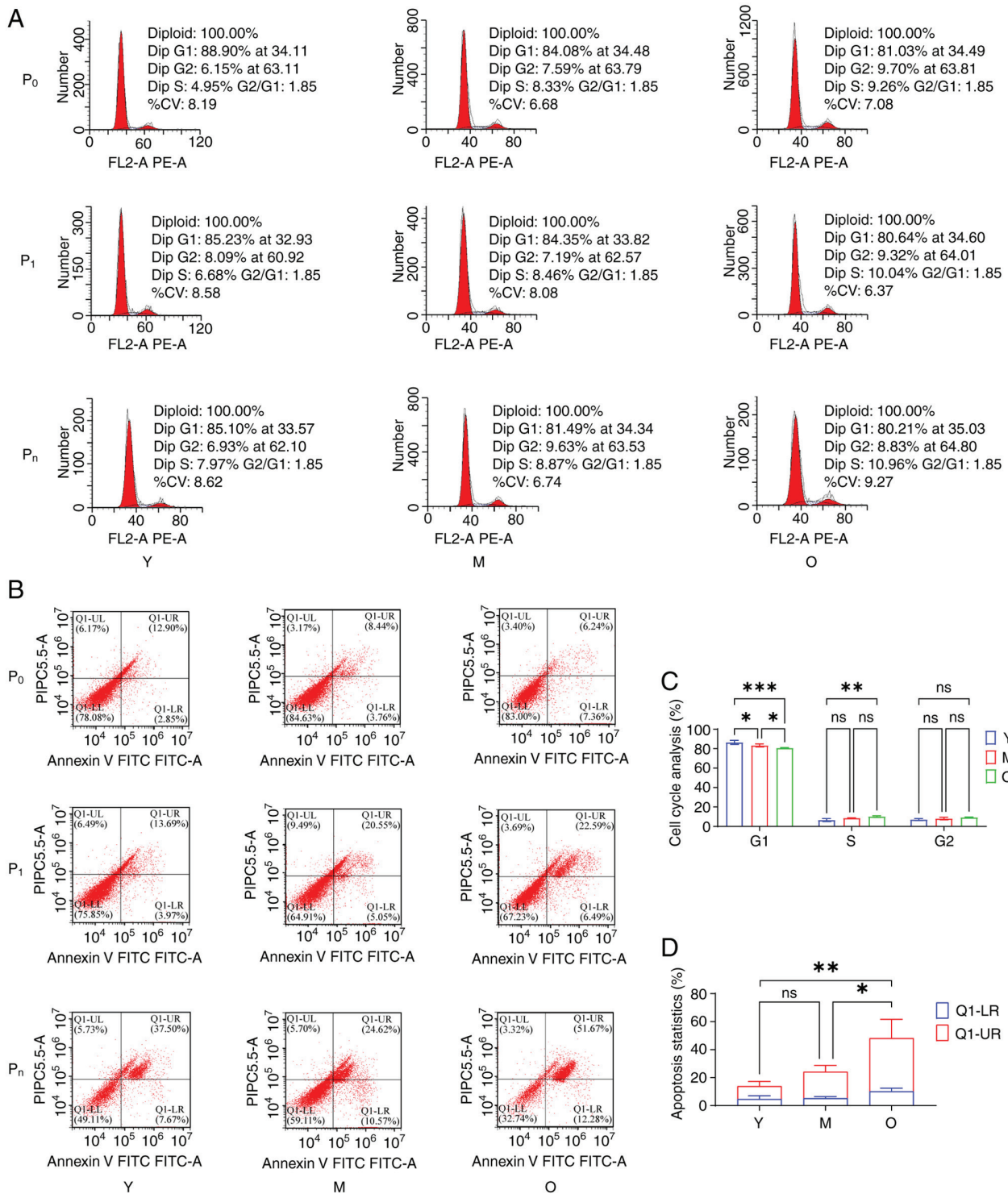


Figure 7. *In vitro* characteristics of bone marrow MSCs. (A) Changes in the cell cycle of bone marrow MSCs at different stages of *in vitro* culture. (B) Apoptotic conditions of bone marrow MSCs at various stages of *in vitro* culture. (C) Statistical analysis of the cell cycle (n=3). (D) Statistical analysis of apoptosis (n=3). *P<0.05, **P<0.01 and ***P<0.001. MSCs, mesenchymal stem cells; ns, no significance.

MSCs transitioned from distinctly spindle-shaped to irregular polygonal forms, characterized by fusion growth (Fig. 8F). These results suggested that changes in the stemness of MSCs are influenced by their growth microenvironment.

To analyze this variability in MSC proliferation, Y group and O group MSCs, which were isolated and purified, were observed using both optical and electron microscopy. Y group MSCs primarily communicated through three types of contact:

‘Synapse-to-synapse’, ‘synapse-to-nuclear zone’ and ‘nuclear zone-to-nuclear zone’. By contrast, O group MSCs exhibited more complex modes of contact due to increased synaptic branching and morphological diversity, with these three forms of cell contact being more pronounced among O group MSCs (Fig. 8G). Electron microscopy results indicated that Y group MSCs had smooth plasma membranes, uniform cell shapes and equal cell body sizes, whilst O group MSCs displayed

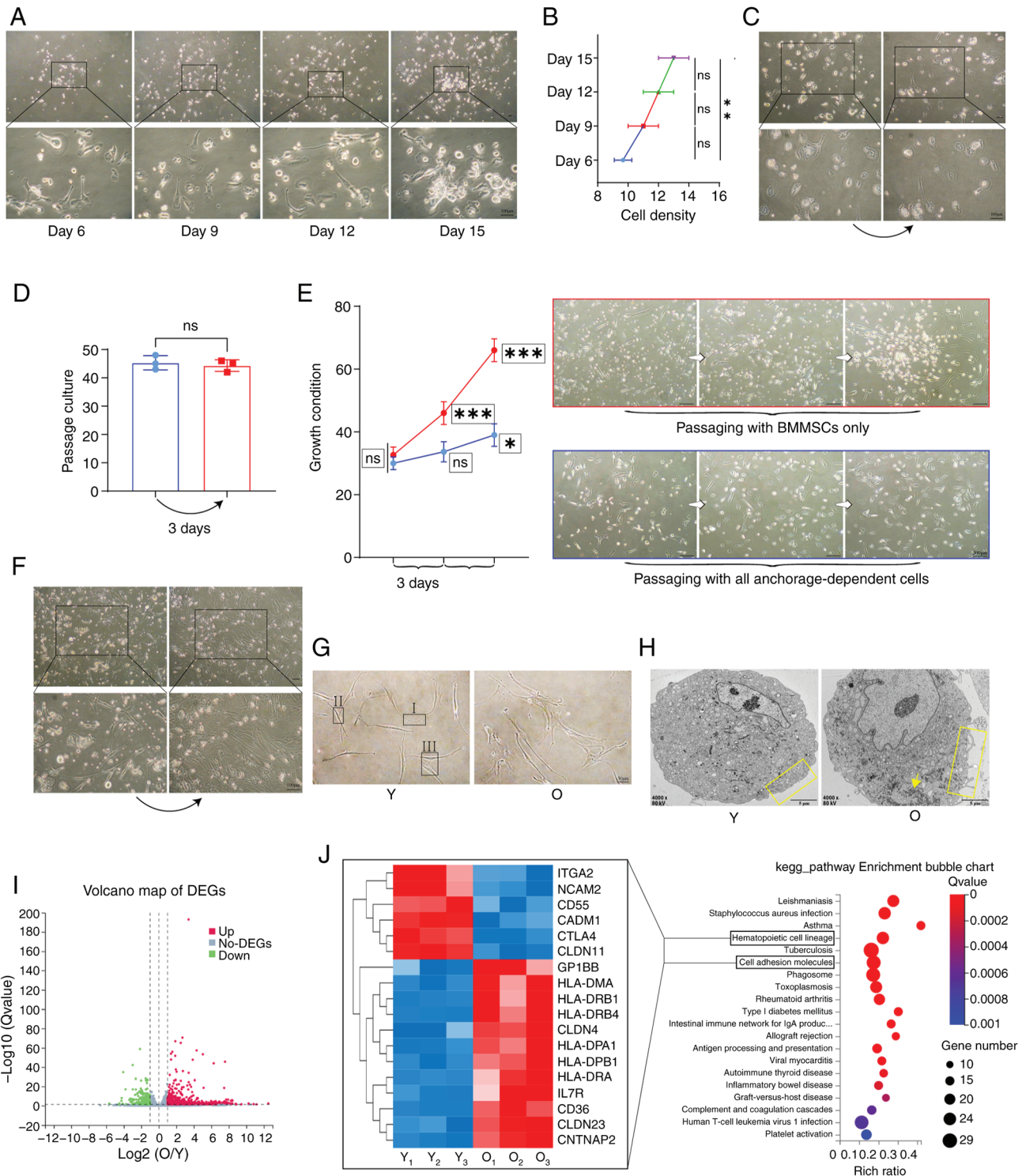


Figure 8. Effects of cell contact on the proliferation of bone marrow MSCs during *in vitro* culture. (A) Adherent proliferation of bone marrow MSCs that were not isolated or purified. (B) Statistical analysis of the *in vitro* proliferation of unpurified bone marrow MSCs (n=3). (C) Passage culture and growth status of adherent bone marrow cells following the removal of bone marrow MSCs. (D) Statistical analysis of the growth status of differentiated adherent bone marrow cells (n=3). (E) Comparison of passage cultures between all adherent bone marrow cells and exclusively isolated and purified bone marrow MSCs, along with their statistical differences (n=3). (F) Growth observation of Y group bone marrow MSCs after co-culture with differentiated adherent bone marrow cells. (G) Cell contact modes of Y group and O group bone marrow MSCs *in vitro*. (H) Structures of MSCs from groups Y and O under electron microscopy. (I) Differential gene volcano map of MSCs from groups Y and O ($|\log_2FC| \geq 1$; Q-value ≤ 0.05). (J) Heatmap of hematopoietic and adhesion-related differential gene clustering in KEGG enrichment methods ($|\log_2FC| \geq 1$; Q-value ≤ 0.05). *P<0.05, **P<0.01 and ***P<0.001. MSCs, mesenchymal stem cells; FC, fold change; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.

irregular membrane protrusions, diverse cell morphologies and increased accumulation of translucent intracellular materials and glycogen (Fig. 8H).

In the transcriptomic analysis, KEGG enrichment analysis of differential genes between Y group and O group MSCs indicated significant differences in hematopoiesis and cell

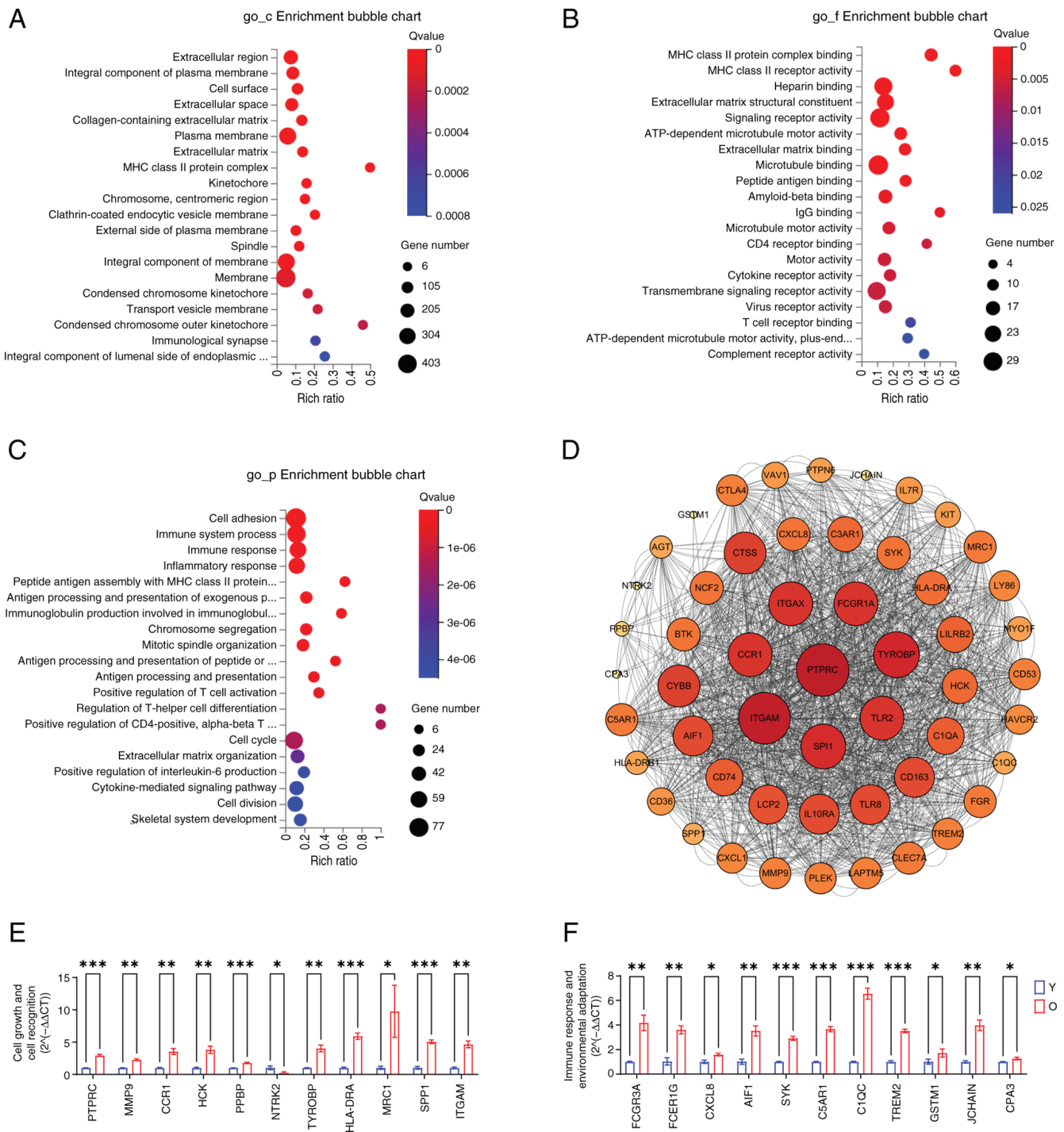


Figure 9. Transcriptomic analysis and validation of Y Group and O group bone marrow MSCs. (A) GO_C analysis indicates significant differences in membrane components between Y group and O group bone marrow MSCs ($\log_2FC \geq 1$; Q-value ≤ 0.05). (B) GO_F analysis suggests that the functional differences in membrane components of Y group and O group bone marrow MSCs are primarily related to cell movement and recognition ($\log_2FC \geq 1$; Q-value ≤ 0.05). (C) GO_P analysis shows that the differences between Y group and O group bone marrow MSCs are reflected in various cellular activities, including cell growth, immune recognition and cell adhesion ($\log_2FC \geq 1$; Q-value ≤ 0.05). (D) Protein interaction networks display the top 53 genes with the greatest variation. (E) Validation of genes related to cell growth and cell recognition (n=3). (F) Validation of genes associated with immune response and environmental adaptation (n=3). *P<0.05, **P<0.01 and ***P<0.001. MSCs, mesenchymal stem cells; FC, fold change; GO, gene ontology.

adhesion. Cluster heat maps revealed distinctions in genes related to hematopoiesis and cell adhesion between the two groups (Fig. 8I and J). GO_cellular component enrichment results suggested that the differences between Y group and O group MSCs are reflected in spatial changes of the plasma membrane and cell membrane transport systems (Fig. 9A). GO_molecular function enrichment results highlighted that

the differences in membrane components predominantly manifested in the expression of cell recognition-related receptors and signaling molecules on membranes (Fig. 9B). GO_biological process enrichment results indicated that these differences are closely associated cell proliferation, adhesion, immune recognition and regulation of bone marrow MSCs (Fig. 9C). PPI analysis displayed the associations of the top 53

differential genes with the greatest variation (Fig. 9D). Finally, RT-qPCR validation results demonstrated substantial differences in cell growth and communication between Y group and O group MSCs (Fig. 9E), with differential validation also observed in genes associated with signaling recognition and immune response (Fig. 9F). These findings suggested that bone marrow MSCs exhibit varying functional stress responses to changes in the bone marrow microenvironment, ultimately adapting their stemness to meet the organism's demands.

Discussion

Hematopoietic stem cell transplantation is one of the primary modalities for treating hematologic malignancies (36,37). Transplanting HSCs in conjunction with bone marrow MSCs can enhance transplantation success rates and promote postoperative hematopoietic reconstruction (38). However, not all transplantation outcomes in clinical settings are favorable. Therefore, in addition to selecting appropriate donors, further investigation into bone marrow MSCs is essential for analyzing strategies to optimally reconstruct the hematopoietic niche (39,40).

Exosomes have emerged as a focal point in the study of bone marrow MSCs (41). The therapeutic effects of exosomes derived from bone marrow MSCs primarily result from their ability to mitigate inflammatory damage (42,43). This phenomenon is closely associated with the low immunogenicity of bone marrow MSCs (44). These attributes make MSCs widely used in regenerative medicine (45,46). This is likely to be one of the key functional manifestations of these cells within the bone marrow microenvironment and a significant factor for successful bone marrow transplantation. Experimental results indicate that the decline in bone marrow function in the elderly is not solely attributable to hematopoietic stem progenitor cells. The reduction in bone marrow MSCs signifies a dysfunction within the bone marrow of older individuals, whilst the formation of numerous terminal bone marrow cells serves as a compensatory mechanism for sustaining bone marrow function (47). The biological differences observed in isolated and purified bone marrow MSCs at various stages of *in vitro* culture are both a consequence of and a contributor to variations in the bone marrow microenvironment (48). Bone marrow MSCs achieve this adaptation through morphological and functional changes. Modifications in cell contact mechanisms and plasma membrane composition allow these cells to efficiently sense the requirements of the bone marrow. The heightened activity of organelles, primarily the endoplasmic reticulum and the increased presence of vesicular materials, suggest that bone marrow MSCs can effectively process signals from the bone marrow microenvironment. Compared with that in the Y group, O group bone marrow MSCs exhibited elevated expression of numerous immune-related genes, with receptors and pathways associated with immune function being activated within the membrane system (49). It has been previously reported that aging hematopoiesis tends to favor myeloid differentiation (50). Therefore, during the aging process, bone marrow MSCs will likely coordinate changes in the bone marrow microenvironment with the hematopoietic functions of hematopoietic stem progenitor cells (51).

The present study examined the relationship among the bone marrow hematopoietic microenvironment, hematopoietic

stem progenitor cells and aging, to elucidate the factors contributing to the decline in hematopoietic function in the elderly. The accumulation of 'abnormalities' within the bone marrow microenvironment during cellular activities diminishes the stemness of stem cells and alters the expression of stemness-related genes. The fate of bone marrow stem cells is influenced not only by the cells themselves but also by their surrounding environment. In treating hematologic diseases in the elderly, the interactions among bone marrow stem cells merit further investigation, particularly compared with the changes resulting from the intrinsic aging of hematopoietic stem progenitor cells (52,53).

Overall, MSCs can assume an important role in bone marrow transplantation therapy and the treatment of hematopoietic disorders, which may provide ideas for novel therapeutic or adjuvant approaches (54). Because of the invasive procedures and ethical requirements of bone marrow aspiration, it was chosen to collect bone marrow from volunteers presenting with symptoms of IDA, in order to exclude the interference of hematologic malignant diseases on the one hand, and mainly because these individuals showed normal results on review after non-radiochemotherapy. Although the anemic samples may be representative of the general population, our approach minimizes the study limitations. The present study only dissected the differential changes in MSCs in young and old individuals from the perspective of an age gradient. Changes in the bone marrow microenvironment are highly complex with multifactorial outcomes. Other perspectives are needed to fully resolve the causes underlying differences in bone marrow function. Differences in the recruitment and migration capacity of MSCs can be performed in future studies, combined with self-renewal capacity assays and multidirectional differentiation capacity assays to assess capability of MSCs in different age groups. In clinical treatment, more effective bone marrow functional recovery can be achieved not only by screening the source of MSCs, but also by interventions to improve the function of the recipient's own MSCs, in addition to by enhancing the stemness of MSCs in transplantation therapy (55,56).

In conclusion, the findings of the present study provide novel theoretical support in utilizing MSCs for the treatment of hematological diseases, especially in bone marrow transplantation. On this basis, the stemness changes of MSCs were analyzed and are expected to establish more effective screening methods and interventions.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The data generated in the present

study may be found in the National Center for Biotechnology Information under accession number PRJNA1183324 or at the following URL: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1183324>.

Authors' contributions

CW and ZY performed all the experimental assays. LW, ZW, JH and YW designed the study. KD and JS analyzed the data. All authors read and approved the final version of the manuscript. CW and YW confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. 2023092) by The Ethics Committee of Chongqing Medical University (Chongqing, China). Written informed consent was obtained from all participants prior to publication of the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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