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Clonal Analysis and Hierarchy of Human Bone Marrow Mesenchymal Stem and Progenitor Cells

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Abstract

Objective—This study was performed to assess adult human bone marrow mesenchymal stem/progenitor cells at a single cell level and to determine a hierarchy based on proliferative potential.

Methods—Adult bone marrow mesenchymal cells expressing the enhanced green fluorescent protein (EGFP) were sorted as single cells into 24-well plates, each well confirmed with single EGFP-positive cells by fluorescence microscopy, and counted every three days. Colonies derived from single cells were expanded then sorted and evaluated using established differentiation protocols for adipogenic, chondrogenic, and osteogenic lineages. Cells were further analyzed by real-time RT-PCR (PPAR γ 2, LEP, LPL, LUM, COMP, BIG, CBFA1, IBSP, BGLAP) and immunocytochemistry (PPAR γ 1/2, Collagen II, Bone Sialoprotein II) specific for tri-lineage differentiation.

Results—Bone marrow mesenchymal cells were found to contain high proliferative potential-mesenchymal colony-forming cells (HPP-MCFC, 7%), low proliferative potential-mesenchymal colony-forming cells (LPP-MCFC, 29%), mesenchymal cell clusters (MCC, 26%), and mature mesenchymal cells (MMC, 38%). All LPP-MCFC, MCC, and MMC colonies reached senescence at the end of the evaluation period. However, HPP-MCFC continued to grow, showed differentiation toward all three lineages, and demonstrated the capacity to give rise to secondary HPP-MCFC upon replating at a clonal level.

Conclusion—These findings suggest that there is a low frequency of bone marrow derived HPP-MCFC that can both self-renew at a single cell level and differentiate toward multiple lineages of mesenchymal origin.

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INTRODUCTION

The hierarchy used for identifying hematopoietic and endothelial progenitors provides a unique quantitative method for assessing the degree of heterogeneity in stem cell cultures and may offer important insights into mesenchymal stem cell (MSC) biology. Hematopoietic high proliferative potential-colony forming cells (HPP-CFC) obtained from both mouse and human bone marrow have been shown to form large colonies (~50,000 cells) in double-layer agar cultures and differentiate into multiple hematopoietic cell types [1–3]. Low proliferative potential-colony forming cells (LPP-CFC) are defined as hematopoietic cells that can grow into colonies smaller than HPP-CFC but greater than 50 cells [3]. HPP-CFC have been further characterized and identified as the most primitive hematopoietic progenitor cells that can be assayed *in vitro* [4]. This paradigm for classifying different cell types based on their proliferative potential has been instrumental in identifying a hierarchy of circulating endothelial progenitor cells (EPC). Ingram et al. [5] identified four distinct cell types in EPC cultures that included high proliferative potential-endothelial colony forming cells (HPP-ECFC), low proliferative potential-endothelial colony forming cells (LPP-ECFC), endothelial cell clusters, and mature differentiated endothelium. HPP-ECFC have been shown to give rise to all subsequent stages of endothelial progenitors as well as secondary and tertiary HPP-ECFC [5].

In this study, the methods used to identify HPP-CFC and HPP-ECFC were adapted to investigate the existence of high proliferative potential-mesenchymal colony forming cells (HPP-MCFC) and the differentiation potential of these cells toward adipogenic, chondrogenic, and osteogenic lineages at a single cell level. This study demonstrates for the first time that a complete hierarchy of mesenchymal cells can be described, and multipotent HPP-MCFC can form secondary colonies at a clonal level.

MATERIALS AND METHODS

Mesenchymal Cell Culture

Human bone marrow mononuclear cells (1×10^7 cells obtained from Cambrex, NJ) from healthy donors (N=3; 1 female and 2 males 25 ± 5 years) were plated on 100 mm plates in α -20 medium (α -MEM supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin) and incubated at 37°C in 5% CO₂. Plates were washed with phosphate buffered saline (PBS) three times every other day until cells reached ~80% confluence (approximately 1×10^6 cells in 100 mm plates). Cells were then washed with PBS and incubated with 0.25% trypsin-EDTA (Gibco, Carlsbad, CA) for 5 min at 37°C, and then replated in α -20 medium at 5×10^3 cells/cm². A bank of cryopreserved mesenchymal cells from each donor was established at the end of the first passage and used for single cell sorting and culturing cell feeders. The overall plating protocol is shown in Figure 1.

Preparation of Mesenchymal Cell Feeders

Since MSC only grow in the presence of other cells an irradiated feeder layer was developed to allow single cell growth and differentiation. Cryopreserved mesenchymal cells were thawed and plated at 5×10^3 cells/cm² in 100 mm plates with α -20 medium and grown to ~80% confluence, as described above. Cells were washed with PBS and incubated with 0.25% trypsin-EDTA for 5 min at 37°C. α -20 medium was added to cells in at least a 1:1 ratio to trypsin in order to inactivate. Cells were collected, washed in fresh medium, and irradiated using a gamma irradiator (J. L. Shepherd & Associates, CA) at 3,000 rads.

Transduction and Sorting

Cryopreserved mesenchymal cells were thawed and transduced with an HIV-1-derived lentiviral vector (1×10^6 infectious particles/ml) expressing the enhanced green fluorescent protein (EGFP) or red fluorescent protein (RFP) under the control of the CMV promoter in medium containing 4 $\mu\text{g/ml}$ polybrene as previously described [6]. Cells were incubated with the vector overnight at 37°C, then washed with PBS and replenished with new medium. After cells reached ~80% confluence, cells were trypsinized and stained with 1 $\mu\text{g/ml}$ propidium iodide. EGFP-expressing mesenchymal cells that did not take up propidium iodide were sorted into 24-well plates with and without irradiated feeder cells at 1 cell/well using a MoFlo Cell sorter (Dako Cytomation, Carpinteria, CA) and incubated overnight at 37°C. On day 1 post-sorting each well was monitored for EGFP-positive cells under a fluorescence microscope equipped with an X–Y stage, and wells containing more than 1 cell were excluded from the study. Wells containing single cells were monitored and counted every 3 days and those that showed growth after 12 days in culture were trypsinized, counted using a hemacytometer, and passaged at 5×10^3 cells/cm². A subset of single fluorescent mesenchymal cell-derived colonies showing >10 population doublings were combined and again sorted into 24-well plates with irradiated feeder cells at 1 cell/well and incubated overnight at 37°C. Wells were verified to contain a single EGFP-positive cell and were monitored for growth for 13 days as described. RFP-positive cells were also cultured with EGFP-positive cells and used as a control to identify any possible cell fusion events.

Differentiation of Mesenchymal Cells

Fluorescent human mesenchymal cells derived from single cells and expanded with irradiated feeders were differentiated towards osteogenic, adipogenic, and chondrogenic lineages using Osteogenic, Adipogenic, and Chondrogenic Differentiation Medium (Lonza, Allendale, NJ) following the manufacturer's recommendations and as previously described [6–8].

PCR Analysis

Total RNA was prepared from sorted EGFP-positive differentiated mesenchymal cells using the Qiagen RNeasy Micro kit (Qiagen, Valencia, CA); cDNA was synthesized using the Sensiscript RT kit (Qiagen). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen) with the primers shown in Table 1. The primers for the EF1 α analysis were EF1 α -Forward (5'-GAC-CCA-CCA-ATG-GAA-GCA-G-3') and EF1 α -Reverse (5'-TGT-GGC-AAT-CCA-ATA-CAG-GG-3'). Amplification was performed in an ABI 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Signal copy numbers were calculated per copies EF1 α based on a standard curve ($r^2 = 0.9997$) [9].

Immunocytochemistry

All primary and secondary antibodies were obtained from Millipore (Billerica, MA). Chamber slides from adipogenic and osteogenic differentiation and sections from chondrogenic pellets were washed for 10 min in PBS then blocked with 1% bovine serum albumin (BSA) in PBS for 20 min. For adipogenic slides, one well was incubated with mouse monoclonal anti-human Peroxisome Proliferator-Activated Receptor (PPAR) $\gamma 1/\gamma 2$ (1:500 dilution) in 1% BSA for 1 h while the other well remained in 1% BSA as a control. Slides were then washed 3 times for 5 min in PBS and incubated for 1 h in goat anti-mouse Alexafluor 594 (Invitrogen, Carlsbad, CA). Osteogenic slides were incubated with rabbit polyclonal anti-human Bone Sialoprotein (BSP) II (1:100 dilution) in 1% BSA in one well and 1% BSA alone in the other for 1 h, followed by 5 min in PBS three times and 1 h of goat polyclonal anti-rabbit Alexafluor 594 (1:200 dilution) incubation. Chondrogenic sections were incubated for 1 h with rabbit anti-human Collagen (COL) II (1:10 dilution), then three 5 min washes in PBS and 1 h with goat anti-rabbit Alexafluor 594 (1:200 dilution) incubation. Control chondrogenic sections were incubated in

1% BSA without primary antibody. All slides were washed three times for 5 min in PBS, mounted with Prolong Gold Antifade and DAPI mounting solution (Invitrogen) and a coverslip placed. Fluorescent images were obtained with the Olympus BX61 microscope (Olympus, Melville, NY).

Flow Cytometry

A total of 2×10^4 mesenchymal cells were suspended in 100 μ l staining buffer (0.1% sodium azide in PBS) and stained with 5 μ l CD14-PE (clone M5E2; BD), CD29-FITC (clone 4B4LDC9LDH8; Beckman Coulter, Fullerton, CA), CD34-PE (clone 563; BD), CD71-FITC (clone DF1513; Ancell, Bayport, MN), CD90-FITC (clone 5E10; BD), and SH3-FITC (American Type Culture Collection, Manassas, VA) antibodies at room temperature for 20 min. 5,000 events were acquired using a FACSCalibur Flow Cytometry System (Becton, Dickinson and Company, Biosciences, San Diego, CA) and analyzed with CellQuest software.

Karyotyping

Trypsinized mesenchymal cells were incubated in 0.05 μ g/ml of colcemid for 20 min at 37°C and then in 0.075 M KCl for 15 min. Cells were fixed in solution containing methanol and glacial acetic acid (3:1). Cells were washed in fixative solution 3 times at 1,200 rpm for 8 min each and submitted to Genzyme (Genzyme Genetics, Orange, CA) for karyotyping.

Statistical Analysis

All studies were performed three times on bone marrow mesenchymal cells obtained from the mononuclear cells from three donors as described above. Results are reported as the mean $\pm$ standard error of the mean (SEM) and calculated using Microsoft Excel (Microsoft, Redmond, WA). Statistical significance ($p < 0.05$) was determined by analysis of variance (ANOVA) or two-sided Student's *t*-test analysis.

RESULTS

Growth of single mesenchymal cells

Single human bone marrow mesenchymal cells expressing EGFP were sorted and cultured with and without irradiated feeder cells for 12 days. EGFP positive cells were counted every three days. Based on population doublings, cells were categorized into four groups: HPP-MCFC (> 9 population doublings, Fig. 2A–D), low proliferative potential-mesenchymal colony forming cells (LPP-MCFC) ($> 6 - 9$, Fig. 2E–H), mesenchymal cell clusters (MCC) ($> 3 - 6$, Fig. 2I–L), and mature mesenchymal cells (MMC) (≤ 3 , Fig. 2M–P). Single cells cultured without cell feeders did not grow beyond 6 population doublings, and a majority of these cells did not divide. Thus, irradiated cell feeders were added in all single mesenchymal cell cultures. Over 12 days in culture, single fluorescent HPP-MCFC proliferated to $1,916.9 \pm 276.9$ cells, which was significantly greater than LLP-MCFC (189.1 ± 14.3 cells), MCC (28.9 ± 2.9 cells), or MMC (2.3 ± 0.3 cells) (Fig. 3; $p < 0.05$). Doubling time of HPP-MCFC (26.2 ± 0.4 h) was significantly shorter than routine heterogeneous adult bone marrow mesenchymal cell cultures (50.2 ± 1.6 h; $p < 0.01$).

All fluorescent mesenchymal cells were sorted into each well and identified as a single cell and formed one of the four distinct colony types based on proliferation as described above. No cell death was observed over time in culture. Regarding frequency, 7% of single fluorescent cells grown on feeders were HPP-MCFC (13 colonies/180 single cells plated); whereas LPP-MCFC, MCC, and MMC occurred at a frequency of 29%, 26%, and 38%, respectively. All LPP-MCFC, MCC, and MMC showed limited proliferative potential and increased cytoplasm by 9 days in culture by visual observation (Fig. 2H, L, P). Specifically, most MMC did not

divide whereas MCC showed 3–6 population doublings and reached senescence after 6 days in culture. ALL LPP-MCFC reached senescence by 12 days in culture. However, HPP-MCFC continued to grow and maintained a spindle-shaped morphology.

Cells were assayed and all found to be karyotypically normal. No significant differences in cell-surface marker expression were observed when cells were grown under routine mesenchymal cell culture conditions [10] and HPP-MCFC were compared by flow cytometry (positive for SH3, CD29, CD71, CD90; negative for CD14, CD34, CD45) (data not shown).

Differentiation of single cells

Fluorescent HPP-MCFC colonies derived from single cells were differentiated toward adipogenic, osteogenic, and chondrogenic lineages and confirmed by Oil-Red O (Fig. 4A), von Kossa (Fig. 4B), and Safranin O (Fig. 4C) staining, respectively. Since these staining methods lead to a loss of fluorescence in single mesenchymal cells, co-localization with EGFP was not possible. These staining methods showed efficient cellular differentiation. To identify single cell-derived mesenchymal cells from the feeder cells when differentiated, cells were stained with antibodies against PPAR γ 1/2 (adipogenic, 4D), BSP-II (osteogenic, 4E), and COL-II (chondrogenic, 4F). Co-localization of EGFP and PPAR γ 1/2, BSP-II, or COL-II in HPP-MCFC was observed in all cultures. To exclude the possibility of cell fusion, EGFP-positive mesenchymal cells were co-cultured with RFP-positive mesenchymal cells. No cell fusion (yellow cells) events were observed in all cell cultures examined (data not shown). Osteogenesis was observed in LPP-MCFC, MCC, and MMC, but no or very low adipogenesis was observed in these cell types (data not shown). A low number of LPP-MCFC was noted to express COL-II in chondrogenic cultures. These results suggest that single cell-derived mesenchymal cells (EGFP-positive) can differentiate into all three lineages investigated in this study.

In addition to immunochemical evaluations we performed a quantitative comparison between EGFP-positive cells (single cell-derived) and feeder cells by qRT-PCR to provide further evidence of the tri-lineage differentiation potential of HPP-MCFC (Fig. 5). Adipogenic, chondrogenic, and osteogenic cultures were dissociated with trypsin and sorted based on EGFP fluorescence. EGFP positive cells (single-cell derived) and negative cells (mesenchymal cell feeders) were analyzed for lineage specific gene expression. An insufficient number of cells was obtained from LPP-MCFC, MCC, and MMC cultures for further cell sorting and PCR analysis. Significantly higher expression of adipogenic genes (PPAR γ 2, LEP, LPL) (Fig. 5A) was observed in EGFP-positive HPP-MCFC compared to mesenchymal cell feeders obtained from the same culture induced with adipogenic factors. Interestingly, no significant differences were observed when the level of chondrogenic gene expression (LUM, COMP, BIG) (Fig. 5B) was compared between HPP-MCFC and the feeders obtained from the same chondrogenic culture. Similar to the adipogenic cultures, higher levels of osteogenic gene expression (CBFA1, IBSP, BGLAP) were observed in HPP-MCFC cultures compared to the cell feeders in the same culture induced with osteogenic factors (Fig. 5C).

Replating potential of HPP-MCFC

To investigate secondary replating potential, we wished to examine individual cells derived from HPP-MCFC colonies for secondary colony formation, however, the average number of HPP-MCFC obtained from single mesenchymal cells was approximately 1,200 cells, which could not be FACS sorted. Thus, HPP-MCFC were pooled for this study, and single cells were sorted then cultured with mesenchymal cell feeders as conducted for the primary studies. Although it is possible that there may be differences between HPP-MCFC colonies, all showed approximately identical proliferative potential as noted providing a rationale for sorting a pooled population to investigate replating potential. As described above for the primary studies,

single fluorescent cells were monitored, and counted for 12 days. A complete hierarchy of cells exhibiting different degrees of self-renewal potential was established in the co-culture. Compared to the initial distribution where 7% of the MSC clones displayed properties of HPP-MCFC, 17% (11 HPP-MCFC/62 single cells plated) of single cells in the secondary co-cultures showed greater than 9 population doublings (Fig. 6). Interestingly, the frequencies of LPP-MCFC and MCC declined to 16% each (10 HPP-MCFC/62 single cells plated) in the secondary cultures. However, the frequency of MMC increased to 51% (31 HPP-MCFC/62 single cells plated) in the secondary co-cultures compared to 38% with the initial sorted clones. Overall, single HPP-MCFC gave rise to 11.0 ± 0.2 population doubling times during the initial expansion, and 10.9 ± 0.3 population doubling times during the secondary plating period, equivalent to a single cell expanding to $3.8 \times 10^6 \pm 6.6 \times 10^5$ cells (Fig. 7).

DISCUSSION

This study demonstrates for the first time that a routine bone marrow mesenchymal cell culture contains a complete hierarchy of cells that can only be assessed by applying a careful single cell analysis. Results also show that single HPP-MCFC can differentiate into multiple mesenchymal lineages and generate secondary colonies of HPP-MCFC. The single cell analysis performed also provides a quantitative view of a population of human bone marrow mesenchymal cells and shows heterogeneity based on growth characteristics. As shown for EPC [11], bone marrow mesenchymal cell cultures contain four distinct cell populations, namely, HPP-MCFC, LPP-MCFC, MCC, and MMC. Although this hierarchy is purely based on growth characteristics, it clearly identifies the degree of heterogeneity of a routine MSC culture. Interestingly, greater than 90% of mesenchymal cells showed limited proliferative potential with nearly 40% of the clones demonstrating less than 3 population doublings prior to senescence. Considering numerous reports indicating that adult bone marrow MSC possess exceptional proliferative potential [10,12,13], these studies indicate that a small subpopulation of cells within a routine bone marrow mesenchymal cell culture may be responsible for establishing a long-term culture. These data suggest that this subpopulation, termed HPP-MCFC, undergoes asymmetric cell division and establishes another generation of mesenchymal cells with varying levels of proliferative potential, that not all HPP-MCFC maintain self-renewal potential, and a pure population of true HPP-MCFC may not be possible to obtain using current culture methods. It is also possible that this asymmetric cell division is a result of suboptimal culture conditions, rather than an intrinsic phenomenon. However, it is important to note that the design of culture conditions used for this study represents those widely used in the field [10].

Another important factor involved in self-renewal of HPP-MCFC is the contribution of mesenchymal cell feeders, which is evident by impaired MSC emergence from single plated HPP-MCFC in a feeder-free culture system. While MSC progeny did emerge in some cultures of single plated MSC, the largest colony of mesenchymal cells that emerged that were feeder free was approximately 70 cells (~6 population doublings). Thus, there is no evidence that a single HPP-MCFC alone can establish its own microenvironment for efficient self-renewal under the culture conditions used in this study. Although it is clear that the optimization of culture conditions is necessary to promote self-renewal of HPP-MCFC without the need for feeders to support the culture, the monolayer of heterogeneous bone marrow mesenchymal cells appears to contain a population of cells that supports self-renewal of HPP-MCFC. Currently, there is no definitive evidence to support the existence of a distinct MSC population and mesenchymal microenvironment in bone marrow. Sacchetti et al. [14] indicated that a single bone marrow CD146+ colony can establish both heterotopic bone and the hematopoietic microenvironment. However, our current data suggests a clear dependency of HPP-MCFC on other cell type(s) in culture for self-renewal. Thus, it is reasonable to postulate that hematopoietic and mesenchymal stem cell populations each may require a different

microenvironment for self-renewal. It is also possible that asymmetric division of HPP-MCFC is contributed to by mesenchymal cell feeders. This hypothesis can further be tested once an optimal culture condition that promotes symmetric cell division is developed.

A study by Aubin et al. [15] showed heterogeneity in osteoprogenitor cell culture when gene expression of individual cells was evaluated by PCR. This investigation provided important insights into functional properties of stem cells that may not be apparent when they are mixed with other cell types. Thus, sub-fractionation of mesenchymal cell culture is crucial in identifying biological properties of true MSC. Colony-forming units (CFUs) obtained by limiting dilution have been used to study the clonality of MSC [12,16–18]. However, it is difficult to show conclusively that each CFU is derived from a single cell since mesenchymal cells tend to adhere to each other. One way to show that each CFU is derived from a single cell is by screening the entire growth surface and confirming that each mesenchymal cell is separate from neighboring cells. Compared to conventional CFU-F, the sorting method of mesenchymal cells used in this study allowed more stringent single cell identification and subsequent screening of individual cells expressing EGFP under the fluorescent microscope. Even under such stringent single cell sorting and culture conditions used in the current study, rare two-cell aggregates were detected microscopically after sorting. These two-cell aggregates showed a tendency for better growth characteristics when compared to single cells and could lead to misinterpretation of growth data. Although it is possible that these cells represented two identical daughter cells, it was not possible to show conclusively that these cells were, in fact, from a single cell except through use of a lentiviral or retroviral insertional analysis at the genomic level. Thus, these two-cell aggregates were excluded from this study. In addition, expression of a fluorescent marker was necessary to conclusively show that each well truly contained a single mesenchymal cell because of the optical edge effect produced by the culture plates. This optical interference occurs mainly at the edge of wells and makes visualization of cells very difficult under bright light, which occurs with CFU-F studies. After sorting, approximately 41% of cells were noted at or very close to the edge of the wells where visualization was difficult due to optical interference. It is important to note that under the optimized lentiviral transduction conditions used in this study no adverse effects such as growth arrest, VSV-G toxicity, and undesired cellular changes [6,19,20] were observed as a result of transduction or EGFP expression. In addition to using EGFP as a marker for sorting single cells and subsequent screening, expression of a functional gene such as alkaline phosphatase has been shown to also be helpful to interrogate the hierarchy of osteoprogenitors that are undergoing differentiation [21]. Purpura et al. used this method to assess cell heterogeneity in fetal rat calvaria cell culture and developed a method to fractionate osteogenic populations useful for investigating differentiation capacities of individual colonies [21]. These studies highlight the importance of evaluating individual cells when attempting to identify the phenotype of true stem cells.

Single cell-derived colonies of HPP-MCFC possess tri-lineage differentiation potential toward adipogenic, chondrogenic, and osteogenic lineages. Other reports have indicated that MSC cultures also contain unipotent and bipotent cells [22,23]. Although it is possible that non-HPP-MCFC identified in the study described here may have these qualities, differentiation assays could not be performed due to their limited proliferative potential. Further studies are necessary to investigate the optimal culture condition(s) to promote self-renewal of HPP-MCFC without the need for feeder cells. As more effort is made to investigate therapeutic properties of MSC in both animal models and humans, studies on the basic biology of these cells and performed at a single cell level will provide important quantitative and qualitative insights into identifying the best source of cells for transplantation.

A population of mesenchymal cells cultured under widely used conditions [10] and commonly considered heterogeneous [13,24] was shown to contain a complete hierarchy of cells

displaying different levels of proliferative potential and HPP-MCFC that can both self-renew and differentiate into three mesenchymal lineages. This study highlights the importance of analyzing mesenchymal cells at a single cell level to identify the presence of true stem cells.

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Figure 1. Plating Protocol

The overall plan for cell plating is shown.

Figure 2. Fluorescence images of mesenchymal cell growth

Images of EGFP expressing cells obtained on day 1 (A, E, I, M) post-sorting, day 3 (B, F, J, N), day 6 (C, G, K, O), and day 9 (D, H, L, P). Four distinct cell populations derived from single mesenchymal cells included high proliferative potential-mesenchymal colony forming cells (HPP-MCFC; A–D), low proliferative potential-mesenchymal colony forming cells (LPP-MCFC; E–H), mesenchymal cell clusters (MCC; I–L), and mature mesenchymal cells (MMC; M–P) which were observed over time in culture. Images shown in each row were taken from the same well over time.

Figure 3. Growth of single mesenchymal cells

Single fluorescent mesenchymal cells expressing EGFP (green, insert) were sorted into 24-well plates with irradiated mesenchymal cells as feeders (EGFP-negative). The insert demonstrates the presence of two EGFP-expressing cells (day 3) within the irradiated feeder cells. EGFP-positive cells were counted every 3 days for 12 days. Wells containing ≥ 2 cells were excluded from the study. Single HPP-MCFC showed a significantly higher growth rate compared to LPP-MCFC, MCC, or MMC ($p < 0.05$). All LPP-MCFC, MCC, and MMC reached senescence, whereas HPP-MCFC continued to proliferate. * $p < 0.05$.

Figure 4. Differentiation of mesenchymal cell clonal colonies

Colonies of fluorescent HPP-MCFC were differentiated toward adipogenic (A, D), osteogenic (B, E), and chondrogenic (C, F) lineages. Adipogenic-differentiated cells were stained with Oil Red-O (red channel only, A, 10X objective) and co-expressed PPAR $\gamma 1/\gamma 2$ (red, D, 40X objective) with the EGFP-HPP-MCFC. Osteogenic-differentiated cells were stained with Von Kossa (red channel only, B, 10X objective) and co-expressed BSP (red, E, 20X objective) with the EGFP-HPP-MCFC. Sections of chondrogenic-differentiated pellets were stained with Safranin-O (red channel only, C, 10X objective) and co-expressed Collagen II (red, F, 20X objective) with the EGFP-HPP-MCFC. No staining (other than the EGFP of the HPP-MCFC progeny) was observed in secondary antibody controls (G–I). Blue = DAPI staining of nuclei.

Figure 5. PCR analysis of differentiated HPP-MCFC

Genes specific for adipogenic (A), chondrogenic (B), and osteogenic (C) lineages were analyzed after sorting EGFP-positive differentiated HPP-MCFC from feeder mesenchymal cells (fdMC, EGFP-negative). Significantly greater levels of adipogenic (A) and osteogenic (C) gene expression were observed in HPP-MCFC compared to fdMC ($p < 0.05$). This effect was not as profound for chondrogenic (B) differentiation when compared to the other two lineages. * $p < 0.05$.

Figure 6. Growth of single mesenchymal cells after sorting

HPP-MCFC from the initial sorting of single cells were pooled, sorted, and monitored for 12 days. The percentage of HPP-MCFC (>9 population doublings) and MMC (≤ 3 population doublings) appeared to increase from the first sort to the second sort, while LPP-MCFC (>6–9 population doublings) and MCC (>3–6 population doublings) were observed to decrease.

Figure 7. Cumulative growth of HPP-MCFC
Single HPP-MCFC proliferated to $3.8 \times 10^6 \pm 6.6 \times 10^5$ cells over 24 days.

Table 1

Primers

Accession #	Gene ID	Gene Name	Forward	Reverse
Adipogenic				
NM 015869.4	PPAR γ 2	Peroxisome Proliferative Activated Receptor, gamma 2	TTGACCCAGAAAGCGATTCC	AAAGTTGGTGGCCAGAATG
NM 000230.2	Lep	Leptin	TGTGCCCATCCAAAAAGTCC	GTTTGGAGGAGACTGACTGCG
NM 000237.2	LPL	Lipoprotein lipase	GCGCCCCCGAGATGGA	TTAGGGCAAAATTACTTTTCGATGTC
Chondrogenic				
NM 002345.3	LUM	Lumican	GGTCAATCAACTTGAGAAAGTTTGACA	GGTGGAAGACTGGTTTCTGAGATG
NM 000095.2	COMP	Cartilage Oligomeric Matrix Protein	CTCCAGCTTCTAGGTGGTCATG	TGGAAGACTTCACAGCCTTGAG
NM 001711.3	BIG	Biglycan	CGGAACATGAACTGCATCGA	TCGAAGGCTCCAGGTTCAAA
Osteogenic				
NM 001024630.2		Run-Related Transcription Factor 2, transcript variant 1		
	RUNX2		CCAAACCAGGAATGCACTATC	TAGTGAGTGGTGGCGGACATAC
NM 004967.3	IBSP	Bone Sialoprotein II	GCATGCCCTACTTTTATCCTCAITTTAA	TCTTCTGAACTGTGTCATCTCCATTTTC
NM 199173.3	BGLAP	Osteocalcin	AAACCCAGCGGTGTCAGAGT	GGCTCCAGCCATTGATACA