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EFFECTS OF PLATING DENSITY AND CULTURE TIME ON BONE MARROW STROMAL CELL CHARACTERISTICS

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Abstract

Objective—Bone marrow stromal cells (MSC) are multipotent adult stem cells that have emerged as promising candidates for cell therapy in disorders including cardiac infarction, stroke and spinal cord injury. While harvesting methods used by different laboratories are relatively standard, MSC culturing protocols vary widely. This study is aimed at evaluating the effects of initial plating density and total time in culture on proliferation, cell morphology, and differentiation potential of heterogeneous MSC cultures and more homogeneous cloned subpopulations.

Methods—Rat MSC were plated at 20, 200 and 2000 cells/cm² and grown to 50% confluency. The numbers of population doublings and doubling times were determined within and across multiple passages. Changes in cell morphology and differentiation potential to adipogenic, chondrogenic, and osteogenic lineages were evaluated and compared among early, intermediate and late passages, as well as between heterogeneous and cloned MSC populations.

Results—We found optimal cell growth at a plating density of 200 cells/cm². Cultures derived from all plating densities developed increased proportions of flat cells over time. Assays for chondrogenesis, osteogenesis and adipogenesis showed that heterogeneous MSC plated at all densities sustained the potential for all three mesenchymal phenotypes through at least passage 5; the flat subpopulation lost adipogenic and chondrogenic potential.

Conclusion—Our findings suggest that the initial plating density is not critical for maintaining a well-defined, multipotent MSC population. Time in culture, however, affects cell characteristics, suggesting that cell expansion should be limited, especially until the specific characteristics of different MSC subpopulations are better understood.

Keywords

adult stem cells; heterogeneity; variability; differentiation potential; expansion

Bone marrow stromal cells (MSC) represent a heterogeneous population derived from the non-blood forming fraction of bone marrow that regulates hematopoietic cell development. *In vitro*, adult mesenchymal stem cells resident in this bone marrow fraction differentiate into bone, cartilage and fat (1). Cultured MSC have also been shown to regenerate cardiac (2) and

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skeletal muscle (3). More recently, it has been suggested that MSC can traverse lineage borders and differentiate into neural cells (for review see (4,5)) as well as epithelia of liver, lung, kidney, skin and the gastrointestinal tract (for review see (6)). This issue, however, remains controversial. Because MSC can be easily obtained using a simple bone marrow aspiration and show an extensive capacity for expansion *in vitro*, these cells have been considered as candidates for cell therapy. So far, MSC have been used with varying success to improve neurological (7–9), cardiovascular (10), blood-related (11,12) and musculoskeletal disorders (13,14).

Isolation of MSC relies on their ability to adhere to plastic. There is no standardized method for culturing MSC; specifically, there are no standards for plating densities, level of confluency and duration of cell expansion. Some studies suggested that plating MSC at low density results in the most rapid proliferation as well as the highest percentage of multipotent cells (15,16). Others have suggested that strict maintenance of very low cell densities throughout expansion is necessary to select for homogeneous cultures of a subpopulation of cells with high proliferation and differentiation potential (17,18).

What is well-recognized is the variability found in MSC cultures. It is obvious that these cultures are composed of different cell types with distinct morphologies. Attempts have been made to classify these subpopulations by marker expression and by shape and growth kinetics, e.g., as spindle-shaped type I or flattened type II cells (19) or as rapidly self-renewing cells, or mature MSC (15,16,20). In addition to the observed heterogeneity *in vitro*, variations have been observed in MSC cultures obtained from different species as well as from different rodent strains or individual human donors (21–23). Some studies (23) found that variations even exist among different MSC donations obtained from the same human donor.

In this context, questions of high clinical relevance remain unanswered: (1) For a given transplantation therapy, what defines the target subpopulation of MSC, and (2) how should this subpopulation be culture-expanded? To resolve this issue, it is important that protocols for cell selection and culture-expansion be devised and tested. It is necessary to use standardized culture protocols that avoid inadvertent amplification of existing variations, or that preferentially expand undesired MSC subpopulations.

In this study, we cultured rat MSC for which no effort was made to limit heterogeneity beyond the base criterion of adherent growth. We call these “heterogeneous MSC,” in distinction to experiments where MSC were cloned on the basis of their morphology. We characterized growth kinetics when seeded at three different densities, and noted the extent to which cultured cells retained the morphology of the culture’s founders. After 5 passages, we also characterized the expanded cells with respect to mesenchymal differentiation potential. Our findings suggest that in heterogeneous MSC cultures, a seeding density of 200 cells/cm² leads to faster growth than much denser or sparser conditions. However, in all conditions the number of flat cells increased over time. In cultures enriched for cloned spindle-shaped cells or flat cells, differences in growth kinetics among different initial plating densities were less obvious. Finally, *in vitro* differentiation assays revealed no difference in the mesenchymal potential relative to initial plating density up to passage 5. However, there was a loss of adipogenic and chondrogenic potential in MSC cloned from flat cells.

MATERIALS AND METHODS

Isolation and culture of rat MSC

MSC were obtained from bone marrow of Fischer rats as described previously (24). Briefly, marrow was flushed from femurs and tibias with Hank’s buffered saline solution (Invitrogen, Carlsbad, CA) and centrifuged at 600 × g for 10 min. Cells were resuspended in 45% Hams’

F-12 (Invitrogen), 45% α -MEM (Invitrogen), 10% fetal bovine serum (HyClone, Logan, UT), and supplemented with 100 U/mL penicillin G and 100 μ g/mL streptomycin sulfate (Invitrogen), then plated into T75 flasks (Corning, Lowell, MA). After 8 days, non-adherent cells were removed and adherent cells were detached with 0.05% trypsin/0.53 mM EDTA (Invitrogen) and replated. After 3 days, cells were detached with 0.1% trypsin/0.02% EDTA (passage 1). MSC were stored in 30% Hams' F-12, 30% α -MEM, 30% serum, and 10% DMSO (Sigma-Aldrich, St. Louis, MO) in liquid nitrogen. For all experiments, MSC were plated at 20, 200 or 2000 cells/cm². Unless otherwise noted, MSC were passaged upon reaching 50% confluency.

Proliferation of MSC over Multiple Passages

MSC were plated in T75 flasks and 35mm dishes and expanded from passage 2 through passage 5 for cells plated at 20 cells/cm² and from passage 2 through passage 10 for cells plated at 200 and 2000 cells/cm². For this expansion experiment, cells were replated at the appropriate seeding density (20, 200 or 2000 cells/cm²) at each passage. Cells were always passaged at 50% confluency, which was an average of every 10.8 days for MSC plated at 20 cells/cm², 5.5 days for cells plated at 200 cells/cm² and 3.1 days for cells plated at 2000 cells/cm². MSC from two 35mm dishes of each plating density were counted each day using a hemocytometer so that cumulative population doubling levels (PDL) could be calculated.

Proliferation in Heterogeneous MSC Cultures

Passage 5 heterogeneous MSC from cultures seeded at 20, 200 or 2000 cells/cm² were replated at 20, 200 and 2000 cells/cm² in 35mm dishes and grown for 14 days without passage with a media change every 3 days.

Cells from each of the growth conditions were counted in duplicate each day. Images were taken each day to document the MSC growth patterns using a Leica DM IRB microscope with phase-contrast optics and Leica DFX 300 FX camera.

Cloning of MSC and Proliferation of Cloned Cultures

Spindle-shaped and flat cells were cloned by limited dilution from passage 2 cultures originally seeded at 200 cells/cm². Briefly, cells were trypsinized, diluted and seeded into 96-well plates (Corning), such that the Poisson distribution gave a 95% probability that all cell-containing wells were originally populated with only a single cell. Visual inspection one day after seeding was used to eliminate wells with more than one founder cell. The mini-colonies that resulted were characterized as containing cells that were either spindle-shaped, flat, or a mix of the two. Spindle-shaped and flat colonies were selected and expanded through wells of 48-well plates and 24-well plates, and into T-75 or T-180 flasks (Corning). After expansion through passage 5, clones were re-plated at 20, 200 or 2000 cells/cm² in 35mm dishes for the 14-day proliferation experiment.

Morphological Classification

The classification of cell type based on morphology was determined by analyzing cell shape and cell processes. In accordance with previous studies (15,16,19,20), two major subpopulations were defined. (a) MSC with a spindle shape had two processes extending in opposite directions from an elongated cell body. This morphology has been termed immature, multipotent, RS1, and RS2 by others (15,16,20). (b) Large cells with a flattened, polygonal shape and a plainly visible nucleus were devoid of processes, or had many short processes. Such MSC had been previously termed "mature with less potential" (15,16,20). In order to assess the effect of initial plating density and time in culture on the morphology of MSC, heterogeneous MSC plated at 20, 200 and 2000 cells/cm² from passages 2, 5 and 10 were

selected. These cells were then plated again at 20, 200 and 2000 cells/cm², respectively, in 35mm dishes and grown until confluency. Each day, images were taken of 20 fields in randomly selected dishes from each group. Cells on these images were counted by 3 independent, blinded observers and categorized as “spindle-shaped” or “flat”. Values were averaged and presented as percentage of spindle-shaped and flat cells.

Differentiation Assays

For differentiation assays, cells from passage 5 from cultures plated at 20, 200 or 2000 cells/cm² as well as cloned populations were used.

Adipogenesis—MSC were seeded into six-well plates at 20,000 cells/cm² and cultured to confluency. Cultures were placed in adipogenic induction medium [DMEM with 4.5 mg/mL glucose (Invitrogen), 10% fetal bovine serum (FBS), 5% rabbit serum (Sigma) and antibiotics, with 0.5 mM methylisobutylxanthine, 1 μM dexamethasone, 10 μg/mL insulin, and 100 mM indomethacin (all Sigma)] for 3 days and subsequently moved to adipogenic maintenance medium [DMEM with 4.5 g/mL glucose, 10% FBS, 5% rabbit serum, antibiotics, and 10 μg/mL insulin] for 1 day. After three cycles, cells remained in maintenance medium for 7 days prior to fixation with 4% paraformaldehyde. Cells were stained with Oil Red O to visualize neutral lipid accumulation.

Chondrogenesis—Chondrogenic assays were performed as previously described (25). 500,000 MSC were pelleted in high-glucose DMEM (Invitrogen) with 1% FBS (Hyclone), antibiotics, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL proline, 2 mM pyruvate (Invitrogen), ITS+ (Collaborative Research, Bedford, MA), 100 nM dexamethasone, 10 ng/mL transforming growth factor-β3 (TGF-β3; R&D Systems, Minneapolis, MN), and 200 ng/mL recombinant bone morphogenic protein-2 (BMP-2; R&D Systems). Medium was changed three times per week. On day 21, pellets were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (5 μm). Some sections were stained with safranin O for detection of anionic proteoglycans. Serial sections underwent immunostaining with a monoclonal anti-collagen II antibody (C4F6; generous gift of Dr. Chichester, University of Rhode Island).

Osteogenesis—MSC were seeded in 6-well dishes at 3,000 cells/cm². After two days, growth medium was replaced by osteogenic medium (including 100 nM dexamethasone (Sigma), 50 μM ascorbate-2-phosphate (Wako Pure Chemicals, Chuo-ku, Osaka, Japan), and 10 mM glycerol phosphate (Sigma)). Medium was replaced twice weekly. After 18 days, medium was aspirated and deposited calcium was solubilized in 0.5 N HCl. Acid-soluble calcium was determined colorimetrically (Sigma Kit 587-M).

The source and treatment of cells for all experiments is outlined in the flow chart in Figure 1.

RESULTS

MSC cultures are heterogeneous

Rat MSC obtained from standard purification methods and maintained in adherent culture consist of a morphologically heterogeneous population. Two major subpopulations can be observed: (a) Cells with an elongated, spindly shape with two processes that extend in opposite directions from the cell body (Figure 2A–C), and (b) polygonal cells with or without short processes (Figure 2D–F). In some previous studies (15, 16, 20), spindle-shaped cells have been classified as “immature,” while polygonal cells have been characterized as “mature.” Colter et al. (15) used flow cytometry to identify smaller spindle-shaped cells as the “RS1” and “RS2” rapidly-cycling, multipotent phenotypes. Since the correlation between cell shape and stem-

cell characteristics has yet to be definitively determined, we describe the two subpopulations simply as “spindle-shaped” and “flat”.

Growth patterns and growth kinetics of heterogeneous MSC depend on initial plating density

MSC were plated at different densities, and expanded through passage 5 (20 cells/cm²) or passage 10 (200 and 2000 cells/cm²), with replicate cultures counted each day. MSC plated at 20 cells/cm² had completed 30.6 population doublings by the end of passage 5 (45 days in culture) with a population doubling time of 35.3 hours. MSC plated at 200 cells/cm² grew much faster, completing 44.9 population doublings over 10 passages (50 days in culture) with a population doubling time of 26.7 hours. However, a further step up in plating density led to decreased growth. Cells plated at 2000 cells/cm² displayed a population doubling time of 40.7 hours over 16.5 population doublings (10 passages; 28 days in culture).

From the perspective of rapidly expanding an initial population of adherent MSC, the plating density of 200 cells/cm² was clearly superior to sparser and denser choices. Cells at this density completed 16 doublings in 65% of the time (19 days) needed for MSC cells plated at higher or lower densities (Figure 3A).

The effect of plating density on MSC growth patterns was also observed during expansion. MSC plated at 20 cells/cm² grew into few large, very dense colonies. The more numerous colonies formed by cells plated at 200 cells/cm² were smaller and less dense. MSC plated at 2000 cells/cm² grew evenly on the plastic (Figure 3B).

Population doubling times vary between heterogeneous and homogeneous cultures

To analyze the differences in growth kinetics between heterogeneous MSC and distinct populations of spindle-shaped or flat MSC, clones were prepared from heterogeneous MSC at passage 2 using the limiting dilution technique. However, distinctions were not absolute. A small percentage of flat cells was observed in populations expanded from a single spindle-shaped MSC. Conversely, small percentages of spindle-shaped cells were observed in populations that had been expanded from a flat MSC founder.

Heterogeneous MSC cultures from passage 5 expansion cultures seeded at 20, 200 and 2000 cell/cm² were plated and grown for 14 days in culture without passage. At the start of the experiment, cells seeded at 20 cells/cm² were at PDL 30.6; cells seeded at 200 cells/cm² were at PDL 19.6, and cells seeded at 2000 cell/cm² were at PDL 6.9.

Spindle-shaped and flat MSC clones were plated at all three seeding densities and also grown for 14 days without passage. At the start of the experiment, the spindle-shaped clones were at PDL 30.9, the flat clones at PDL 27.2 (Table 1).

Heterogeneous MSC cultures exhibited a log phase that lasted 5, 4 and 4 days for MSC plated at 20, 200 and 2000 cells/cm², respectively. The population doubling times exhibited by these cultures during the log phase were 16.2 hours for those plated at 20 cells/cm², 15.2 hours for cells plated at 200 cells/cm² and 16.6 hours for cells plated at 2000 cells/cm². During the two weeks allotted to this experiment, MSC plated at 20 cells/cm² completed 10.5 population doublings, cells plated at 200 cells/cm² underwent 8.3 population doublings, and MSC plated at 2000 cells/cm² had a total of 6.0 population doublings (Figure 4A, Table 1).

Cloned cells grew at slower rates. Spindle-shaped MSC clones plated at 20 cells/cm² proliferated in the log phase from day 1 through day 7 at an average population doubling time of 20.7 hours, completing 9.5 population doublings over 14 days. Spindle-shaped clones plated at 200 cells/cm² showed exponential growth from day 1 until day 7 with an average population doubling time of 22.1 hours reaching approximately 8.1 population doublings. Spindle-shaped

MSC clones plated at 2000 cells/cm² demonstrated exponential growth until day 4 and had an average population doubling time of 24.0 hours, completing only 4.8 population doublings over 14 days in culture (Figure 4B, Table 1).

The population doubling time of flat MSC plated at 20 cells/cm² was 24.8 hours during the log phase and reached the stationary phase at day 9. These MSC completed 9.3 population doublings over 14 days. Flat MSC plated at 200 cells/cm² exhibited a doubling time of 25.6 hours during the log phase, entered the stationary phase on day 8 and completed approximately 8.9 population doublings by day 14. The log phase of MSC flat clones plated at 2000 cells/cm² lasted until day 5. During the log phase flat clones had a population doubling time of 20.3 hours and the total number of population doublings was 6.9 (Figure 4C, Table 1).

Time in culture increases percentage of flat cells independent of initial plating density

In order to determine how plating density, time in culture and passage affect MSC morphology, heterogeneous MSC from passages 2, 5 and 10 were plated at 20, 200 and 2000 cells/cm² and imaged each day until cells were confluent. MSC were classified as either spindle-shaped or flat; data for each phenotype is presented as the percentage of total MSC (Figure 5). Data are presented from day one after plating, from the day on which cultures were 50% confluent, and from the first day cells were confluent.

MSC from passage 2 showed an 80% or better percentage of spindle-shaped MSC on the day after plating at all three seeding densities. The majority of MSC plated at 20 cells/cm² presented a flat morphology on day 7. MSC plated at 200 cells/cm² maintained a 60% majority of spindle-shaped cells at day 5, although flat cells had come to dominate the culture (80%) by the time confluence was achieved on day 9. Throughout the culture of the most densely seeded plates (2000 cells/cm²), spindle-shaped cells dominated.

When later-passage MSC were used to found the cultures, a higher proportion of flat cells was generally present. At passage 5, 70% of MSC plated at all three densities were spindle-shaped on day 1. By 50% confluence, that percentage had slipped to between 40% and 50% for the three densities. In all cases, by the final day of imaging less than 10% of MSC displayed spindle-shaped morphology.

Finally, at passage 10, 80% of the MSC plated at 20 cells/cm² were spindle-shaped on day 1, whereas MSC plated at 200 and 2000 cells/cm² consisted of approximately 50% spindle-shaped cells. Similar to passage 5 MSC, fewer than 20% of cells were spindle-shaped by the time the cultures attained confluence.

Taken together, these data suggest that over time, regardless of plating density, the proportion of spindle-shaped MSC decreased.

The history of plating density does not affect mesenchymal potential

To determine if the mesenchymal differentiation potential of MSC was altered by plating density or by our cloning procedures, chondrogenic, adipogenic and osteogenic differentiation assays were used to induce differentiation in heterogeneous MSC from passage 5 plated at 20, 200 and 2000 cells/cm², and in cloned populations. In the chondrogenesis assay, increased pellet size, Type II collagen secretion, and positive Safranin O staining indicates differentiation. Chondrogenesis was evident in MSC plated at all three densities, as shown by positive Safranin O staining (Figure 6A–C). The pattern of Type II collagen staining corresponded with Safranin O (data not shown). MSC that had consistently been plated at 20 and 200 cells/cm² produced pellets greater than 2 mm in diameter (Figure 6A,B), and those plated at 2000 cells/cm² produced a pellet greater than 1 mm in diameter (Figure 6C). Spindle-shaped MSC produced a similarly-sized pellet and were positive for Safranin O staining (Figure

6D). Only flat MSC produced a pellet without evidence of chondrogenesis (Figure 6E). Thus, high initial plating density does not appear to hinder the ability of MSC to undergo chondrogenic differentiation. However, flat MSC appear to have diminished capacity for chondrogenesis.

The ability to differentiate to fat was evaluated by analysis of Oil Red O staining after induction with adipogenic medium. MSC maintained in regular growth medium were used as controls; all control wells were negative for Oil Red O staining. Surprisingly, MSC plated at 20 cells/cm² were also negative for Oil Red O staining (Figure 6F). These assays may have failed because MSC did not reach confluency, which is normally required for adipogenic differentiation. MSC plated at 200 and 2000 cells/cm² were positive for Oil Red O staining (Figure 6 G,H).

When cloned MSC were assayed for adipogenesis, spindle-shaped MSC clones showed positive Oil Red O staining (Figure 6I). In contrast, flat MSC were negative for adipogenesis (Figure 6J). Thus, the adipogenic potential of flat MSC appears to be limited.

Osteogenesis of MSC was evaluated by scoring calcium deposition after induction with osteogenic medium. Approximately 20 µg of calcium per confluent 35-mm dish is indicative of low levels of mineralization, similar to that which has been observed with fibroblasts. Untreated MSC display about 30 µg calcium. The production of over 40 µg of calcium is considered a positive result in this *in vitro* assay. All tested cultures yielded calcium deposition greater than 40 µg in response to treatment with osteogenic medium. MSC from passage 5 plated at 20 cells/cm² yielded 53 µg, at 200 cells/cm² 46 µg and at 2000 cells/cm² 43 µg calcium. Spindle-shaped and flat MSC both yielded 54 µg calcium.

DISCUSSION

MSC are attractive candidates for transplantation therapies because they can be easily harvested and expanded, they have the potential for autologous transplantation, they appear to “home” to the site of injury (26,27) and they do not carry the ethical burden of embryonic stem cells. To date, studies using MSC in different disease/injury models have yielded varying results. Variability may reflect differences between individual donors (or donor species). However, another confounding factor for this variability may be the differences in culturing and expansion methods used by different investigators. Initial plating densities, level of confluency at passage and harvest, and time in culture are factors with unclear impact on therapeutic efficacy. Since MSC are selected only by the ability to grow in an adherent fashion under the conditions selected by the investigator, they need not comprise a homogeneous population at the outset of *ex vivo* expansion. Differences in culture conditions could further augment existing variations or, alternatively, could select for specific subpopulations or drive subpopulations towards a specific fate. At this point, most researchers believe that the small spindle-shaped population represents the multipotent subpopulation, so this is the population that is considered desirable for cell therapy. Some protocols strive to select for these cells in culture (15,16,28). However, there is no clear evidence that this specific population is more efficacious in animal models of disease and injury. Multipotency may not be the determining factor as the beneficial effect of MSC may rely on secretion of therapeutic factors and regulation of expression of extracellular matrix molecules. Furthermore the identity of the desirable subpopulation may depend on specifics of disease and therapy.

The aim of this study was to evaluate a range of growth protocols with respect to effects of initial plating density and time in culture on growth kinetics, on the levels of heterogeneity within the cultures, and on *in vitro* differentiation. We were able to expand MSC at least to 45 PDL. We found that initial plating density affects growth patterns and kinetics, but does not

significantly affect the differentiation potential of heterogeneous MSC populations. Time spent in culture, however, was a critical factor, affecting the appearance of flat cells. Characteristics of cloned spindle-shaped and flat MSC did not differ notably from those of heterogeneous cultures with the exception of a diminished differentiation potential in flat MSC. Doubling times, however, were not appreciably different between spindle-shaped clones and flat clones.

Previous studies have suggested that low initial plating densities result in higher yields and faster expansion of MSC (15,16). Cells plated at low densities yielded more doublings per passage, as growth at higher densities becomes constrained by density-dependent growth inhibition. In our hands, MSC plated at 200 cells/cm² had the fastest doubling times. A potential reason may be that, in contrast to previous studies (15,29), we did not grow cells to confluency. Instead, we passaged them while still in log phase. The level of confluency clearly influences growth kinetics and selection of subpopulations (17,18).

The growth patterns of MSC cultures also depended on the initial plating densities. At low plating density, MSC grew as very dense colonies, whereas at high plating density, MSC spread evenly across the plate. At intermediate density, the growth pattern was a mix of colonies, albeit not as densely clustered as the ones observed at low plating density, and single cells dispersed across the plate. The difference in growth patterns can certainly affect growth kinetics. In the dense colonies observed at low plating density, cell growth is likely to be inhibited at the colony center because of contact inhibition. Conversely, the sparse single-cell distribution observed at high plating density may not have provided sufficient stimulus for growth. Gregory et al (30) suggested that MSC differentiation is affected by the microenvironment presented both at the center of dense colonies and in the sparse colony periphery. Similar mechanisms may also regulate cell proliferation. Plating cells sparsely without a program of early reseeded to avoid overcrowding at the colony center may affect proliferation directly or indirectly through the induction of early differentiation of MSC. In either event, plating cells at intermediate densities and passaging them frequently should avoid this issue.

We found that the number of flat cells increased over time in all cultures independent of plating density. Since cloned populations of flat MSC lost adipogenic and chondrogenic potential, it is possible that the flat phenotype represents MSC with less potential. The transition between spindle-shaped and flat cells could be due to cell-cell contact, or to exposure to factors in the culture medium. Since flat cells were found within colonies, at the periphery of colonies, and in evenly spread cultures, and since they appeared in higher number towards the end of each passage, it seems plausible that paracrine or autocrine factors secreted into the medium are responsible for the transition. This is also supported by the observations that very sparse cultures that are passaged frequently at low density (17,18) and cultures expanded long-term at consistently high density (20) remain mostly homogeneous, spindle-shaped in one case and flat in the other case. In support of the notion that flat cells represent MSC with less potential, the latter culture was also shown to lose differentiation potential, similar to our observation with flat MSC clones (20). Interestingly, we always found a small number of spindle-shaped cells in cultures founded by flat MSC. Clearly, flat MSC are not terminally differentiated, as they continue to undergo cell division. It is not clear if these spindle-shaped cells represent recently divided flat MSC that will shortly re-acquire the flat phenotype, or if they are a subpopulation that can be re-derived from flat MSC.

We found no effect of initial plating density on differentiation potential, which is in contrast to previously published data (16). This may be due to differences in the level of confluency.

It may prove impractical to fully control MSC heterogeneity and variability with standardized procedures that can be realistically implemented in GMP facilities. Still, it remains important

to identify the phenotypic characteristics that are most reflective of MSC performance. As this is accomplished for human and animal MSC, the confidence in the predictive power for evaluating MSC therapies in animal models of disease will increase. Equally, these insights should open the way towards identification of surrogate biomarkers. In some cases, the use of uncultured marrow to entirely avoid the changes in MSC wrought by *in vitro* culture may be necessary.

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Figure 1. Experimental design

Figure 2. MSC cultures contain morphologically distinct cell populations

Micrographs depict MSC harvested at one time from the same rat. (A–C) A subpopulation of MSC exhibit elongated, spindle-like shapes. (D–F) A second subpopulation of MSC has flattened polygonal morphologies with short or no processes.

Figure 3. MSC plating density effects growth kinetics across multiple passages

MSC were plated at 20, 200 and 2000 cells/cm², passaged at 50% confluency and counted at each passage in order to analyze the effect of plating density on rate of proliferation. A) Cumulative PDL and doubling times were calculated. B) Micrographs depict growth patterns of MSC plated at 20 cells/cm² (top), 200 cells/cm² (middle) and 2000 cells/cm² (bottom). Cultures plated at high density were spread evenly throughout the dish, whereas cells plated at lower densities grow in colonies. Pictures are representative of cultures one day after plating (left column), mid-way through culture (center column) and on the day of passaging at 50% confluency (right column).

Figure 4. Growth kinetics of heterogeneous cultures and cloned spindle-shaped and flat cultures
Heterogeneous MSC from passage 5, and cloned populations of spindle-shaped and flat MSC were plated at 20, 200 and 2000 cells/cm², expanded for 14 days and counted in duplicate on each day to determine growth rate. Cumulative PDL and doubling times were calculated for heterogeneous MSC (A), spindle-shaped clones (B) and flat clones (C).

Figure 5. Proportion of MSC with flat morphology increases over time in culture regardless of plating density

MSC were plated at 20, 200 and 2000 cells/cm², counted daily until confluent and classified as spindle-shaped or flat. Data is presented as percentage of total MSC counted on day one after plating (d1), on the day cultures reached 50% confluency (d-50%) and on the day after cultures reached 100% confluency (d-100%). While percentages vary between conditions, the proportion of MSC with flat morphology in heterogeneous cultures increases within one passage over time in culture, as well as with increased passage number. The time elapsed from seeding to cultures achieving 50% or 100% confluency varied according to plating conditions.

Figure 6. Chondrogenic and adipogenic potential is limited in MSC with flat morphology, but is unaffected by plating density

MSC plated at different densities as well as cloned spindle-shaped and flat populations were subjected to chondrogenesis (A–E) and adipogenesis (F–J) assays. MSC plated at 20 (A), 200 (B) and 2000 cells/cm² (C) demonstrated high levels of chondrogenesis evident by medium or large size pellets and positive Safranin O staining. Spindle-shaped MSC clones also successfully differentiated in the chondrogenic assay as indicated by the large pellet with positive Safranin O staining (D), whereas the flat MSC population did not differentiate under chondrogenic conditions (E). MSC plated at 20 cells/cm² showed no Oil Red O staining in the adipogenesis assay (F), but this may have been due to a limitation of the assay in culture with

low cell density. MSC plated at 200 (G) and 2000 cells/cm² (H) were positive for Oil Red O staining, indicating adipogenic differentiation. The cloned population of spindle-shaped MSC was positive for Oil Red O staining (I); however, cloned flat MSC showed no evidence of adipogenic differentiation (J).

Table 1

Growth of MSC at Passage 5 for 14 days.

MSC	seeding (cells/cm ²)	starting PDL	log phase duration	log phase dbl. time	doublings (log phase)	doublings (14 days)
20 cells/cm ²	20	30.6	5 d	16.2 hr	7.4	10.5
200 cells/cm ²	200	19.6	4 d	15.2 hr	6.3	8.3
2000 cells/cm ²	2000	6.9	4 d	16.6 hr	5.8	6.0
Spindle clone	20		7 d	20.7 hr	8.1	9.5
	200	30.9	7 d	22.1 hr	7.6	8.1
	2000		4 d	24.0 hr	4.0	4.8
Flat clone	20		9 d	24.8 hr	8.7	9.3
	200	27.2	8 d	25.6 hr	7.5	8.9
	2000		5 d	20.3 hr	5.9	6.9