Stem Cell Research (2013) 11, 1365–1377

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Onset of heterogeneity in culture-expanded bone marrow stromal cells

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Received 14 May 2013; received in revised form 10 September 2013; accepted 11 September 2013
Available online 21 September 2013

Abstract Inconsistencies among in vitro and in vivo experiments using adult mesenchymal stem cells (MSCs) confound development of therapeutic, regenerative medicine applications, and in vitro expansion is typically required to achieve sufficient cell numbers for basic research or clinical trials. Though heterogeneity in both morphology and differentiation capacity of culture-expanded cells is noted, sources and consequences are not well understood. Here, we endeavored to observe the onset of population heterogeneity by conducting long-term continuous in vitro observation of human adult bone marrow stromal cell (BMSC) populations, a subset of which has been shown to be stem cells (also known as bone marrow-derived MSCs). Semi-automated identification and tracking of cell division and migration enabled construction of cell lineage maps that incorporated cell morphology. We found that all BMSCs steadily grew larger over time; this growth was interrupted only when a cell divided, producing two equally sized, morphologically similar daughter cells. However, a finite probability existed that one or both of these daughters then continued to increase in size without dividing, apparently exiting the cell cycle. Thus, larger BMSCs are those cells that have exited the normal cell cycle. These results hold important implications for MSC in vitro culture expansion and biophysical sorting strategies.

Introduction

Adult human mesenchymal stem cells (MSCs) are multipotent cells that have been isolated from various tissues and differentiated in vitro into multiple mesodermal lineages such as osteoblasts, adipocytes, and chondrocytes (Caplan, 1991). These cells offer certain practical advantages over embryonic stem cells for therapeutic use, as adult human MSCs can be self-donated (Hare et al., 2012), have exhibited lower risk of teratomas (Knoepfler, 2009), and are not subject to the same ethical issues (Zomorodian and Baghaban Eslaminejad, 2012). Bone marrow stromal cells (BMSCs), a subset of which has been shown to be stem cells (also known as bone marrow-derived mesenchymal stem cells) are currently in clinical trials for graft versus host disease (GVHD), and are widely studied for both tissue repair and immune therapies. However, BMSC-based therapies in humans have produced inconsistent results that have been attributed to donor-to-donor variability (Siddappa et al., 2007), differing isolation/culturing protocols (Seeger et al., 2007), and functional heterogeneity within primary cell cultures or clonal populations of bone marrow-derived MSCs (Muraglia et al., 2000).

Abbreviations: MSC, mesenchymal stem cell; BMSC, bone marrow stromal cell; GVHD, graft versus host disease.

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http://dx.doi.org/10.1016/j.scr.2013.09.004

The inability to isolate pure populations of MSCs for therapeutic applications has largely stemmed from the lack of effective and reproducible methods to isolate MSCs; these cells do not express sufficiently specific biomolecular surface markers (Halfon et al., 2010; Pevsner-Fischer et al., 2011; Oswald et al., 2004). Conventional procedures for isolating MSCs for research and clinical applications frequently rely on in vitro selection of plastic-adherent mononuclear cells from the bone marrow. While prospective isolation of bone marrow-derived MSCs based on the minimal criteria for defining human MSCs proposed by the International Society for Cellular Therapy (Dominici et al., 2006) has helped minimize differences among laboratories, the list of proposed biomolecular markers alone does not strictly characterize MSCs (Bianco et al., 2008). Therefore, researchers have relied on in vitro potency assays to evaluate and identify MSCs; the inadequacies of such approach are that it is both retrospective and does not reliably predict cell functions in vivo. For example, it has been reported that in vitro osteogenic differentiation assays may not reflect the ability of MSCs to form heterotopic osseous tissues in vivo (Kuznetsov et al., 1997). Additionally, a recent study on GVHD patients administered with MSCs revealed no correlation between the ability of the MSCs to suppress T cell proliferation in vitro and clinical efficacy in vivo (von Bahr et al., 2012).

Another approach to identify MSCs is quantitative comparison of physical or mechanical characteristics of the cells (Maloney et al., 2010; Darling et al., 2008). Specifically, different morphologies have been used to describe MSCs cultured in vitro. MSCs have been described as fibroblastoid cells (Kuznetsov et al., 1997), spindle shaped cells (Xu et al., 2004), and a combination of very small round cells and flattened enlarged cells (Colter et al., 2001). The smaller cells are typically considered as more "naive" and capable of tri-lineage differentiation, while the larger cells have been reported to be more mature and restricted in differentiation potential (Colter et al., 2001). It has been observed in clonal cultures of BMSCs that smaller cells tend to reside at the periphery while the larger cells are more prevalent in the colony center (Ylostalo et al., 2008). Several studies have explored the source of this morphological and functional heterogeneity, and attributed this variously to: (1) reprogramming upon removal from the in vivo marrow niche (Zipori, 2010); (2) increased mutation probability due to high oxygen tension under in vitro culture conditions (Wagner et al., 2010); (3) stochastic or deterministic changes in the rate of replicative senescence (Wagner et al., 2010); (4) differences in commitment and maturation within the population (Ratajczak et al., 2008); (5) cell cycle stage (Lee et al., 2011); and (6) variation in culture conditions among many cells in a single tissue-culture dish (Bruder et al., 1997). Some studies point to cell plating density or cell–cell contact as the main contributor to changes in cell size and morphology (Ylostalo et al., 2008; Colter et al., 2000). However, other reports have demonstrated that cell density has no significant effect on MSC phenotype (Haack-Sørensen et al., 2012) and that small cells have been observed to arise in cultures that started with only large cells, and vice versa (Neuhuber et al., 2008). Therefore it is, to date, unclear how putative MSCs that exhibit different sizes and morphologies within BMSC cultures are related and may differ functionally.

Although it is anecdotally established that the size of attached BMSCs increases over time and that more of these larger cells are observed with increasing passage number, past observations are based on static observations of culture or are population based over time. Several questions have not been fully answered: How do the differences in cell size arise? Does the original cell population include subpopulations that change in proportion over time? Can smaller cells become larger cells, and vice versa? Do BMSCs divide asymmetrically to make some daughter cells larger than others? Do small cells stay small upon division, to maintain a distinct subpopulation, but then proliferate faster to the point of senescence and disappearance from the culture at early passages (Colter et al., 2001)?

An approach that has shown promise for answering these types of questions in other stem cell systems, and is becoming increasingly feasible with advanced computing power and improved tracking algorithms, is long-term in vitro imaging of individual stem and progenitor cells (Schroeder, 2011). Long-term imaging can enable the construction of cell lineage maps, tracking the progression from parent cell to daughter cells and revealing the emergence of properties of interest. For example, long-term imaging has provided data to detail the complex commitment hierarchy of embryonic (Ravin et al., 2008) and adult (Costa et al., 2011) neural stem cells, successfully captured instances of hemogenic endothelial cells giving rise to blood cells (Eilken et al., 2009), and allowed the comparison of cell cycle time, migration speed, and growth kinetics between hematopoietic stem cell siblings (Scherf et al., 2012). Here, we utilize long-term in vitro imaging to address some of the outstanding questions regarding cell-size heterogeneity in commercially purified, culture-expanded BMSCs.

We demonstrate changes in size (suspended cell diameter and adherent cell spread area) of adult human BMSCs across in vitro passages. We then quantify the onset of heterogeneity in cell size and division rate among BMSCs during extended timelapse imaging in vitro within a single passage. We construct cell lineage trees showing both the timing of cell division and cell size over time for descendants from an initial population of cells. We can thus ascribe a generational "age" to individual cells based upon the number of cell divisions since the start of the observations, so that "younger" generation cells have undergone fewer previous divisions than their "older" counterparts. Additionally, we calculate cell "lifetime" as the time from when cell A first splits (from its sister cell B to complete the mitotic event) to when cell A begins to divide itself (giving rise to its own pair of daughter cells). We find that large cells are not necessarily either younger or older cells than their smaller counterparts, but rather are cells from any generation that have stopped dividing. These findings quantify and identify the source of size-based heterogeneity within in vitro BMSC populations, which can enable culture standardization and may allow for more effective purification of bone marrow-derived mesenchymal stem cells.

Materials and methods

Cell culture

Cells from multiple commercial sources were used. We refer to these cells as human bone marrow stromal cells (BMSCs), though