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Induction of quiescence (G0) in bone marrow stromal stem cells enhances their stem cell characteristics

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ABSTRACT

Several studies have suggested that bone marrow stromal steam cells (BMSC) exist in a quiescent state (G0) within the in vivo niche; however, an explicit analysis of the biology of G0 state-BMSC has not been reported. We hypothesized that induction of G0 in BMSC might enhance their stem cell properties. Thus, we induced quiescence in BMSC in vitro by (a) suspension culture in a viscous medium or (b) culture on soft polyacrylamide substrate; and examined their molecular and functional phenotype. Induction of G0 was confirmed by bromodeoxyuridine (BrdU) labelling and analysis of cell cycle gene expression. Upon reactivation and re-entry into cell cycle, G0 state-BMSC exhibited enhanced clonogenic self-renewal, preferential differentiation into osteoblastic rather than adipocytic cells and increased ectopic bone formation when implanted subcutaneously in vivo in immune-deficient mice, compared to asynchronous proliferating (pre-G0) BMSC. Global gene expression profiling revealed reprogramming of the transcriptome during G0 state including significant alterations in relevant pathways and expression of secreted factors, suggesting altered autocrine and paracrine signaling by G0 state-BMSC and a possible mechanism for enhanced bone formation. G0 state-BMSC might provide a clinically relevant model for understanding the in vivo biology of BMSC.

1. Introduction

Cellular quiescence (G0) is an intrinsic property of adult stem cells (ASC) in vivo that allows suppression of cell division and tissue-specific genetic programs without affecting the capacity for cell cycle re-entry and subsequent differentiation (Cheung and Rando, 2013, Subramaniam et al., 2013, Rumman et al., 2015). Earlier studies demonstrated that bone marrow stromal cells (BMSC) in vivo display characteristics of G0 state, as they are label-retaining and resistant to 5-fluorouracil (5-FU)-induced apoptosis (Haas et al., 1969). More recently, a subset of label-retaining cells was identified in mouse periosteum and found to co-express BMSC markers (Cherry et al., 2014). In addition, subpopulation of freshly isolated PDGFRα(+) SCA-1(+) murine BMSC were found to be in G0 state as assessed by DNA and RNA content (Morikawa et al., 2009). However, information regarding the functional characteristics of G0 BMSC has not been reported.

BMSC (also known as bone marrow skeletal stem cells or mesenchymal stem cells) represent a population of plastic adherent cells isolated from bone marrow aspirates (Zaher et al., 2014, Bianco and Robey, 2015) and exhibit regeneration-enhancing characteristics upon transplantation in a number of in vivo disease models such as bone

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fracture (Granero-Molto et al., 2009), ischemic heart disease (Cai et al., 2016), liver injury (Wang et al., 2016). However, little is known about the biology of G0 BMSC, and many inferences are based on studies of BMSC in culture may not be appropriate since proliferating plastic-adherent cells differ in key regulatory properties from the G0 state that predominates in adult stem cells in vivo (Morikawa et al., 2009; Li et al., 2014).

As a first step in understanding the biology of quiescent BMSC, we examined the effect of inducing the G0 state on stem cell and differentiation functions in culture. In a number of cellular models, induction of G0 causes significant changes in cellular and molecular functions of the cells (Pallafacchina et al., 2010; Mourikis et al., 2012). Thus, we induced G0 in BMSC using two approaches: suspension culture in methylcellulose (MC) and culture on soft polyacrylamide (PAA) substrates and we examined the impact on BMSC stemness and changes in transcriptome as compared to cultured asynchronous proliferating (pre-G0) BMSC. Our results demonstrate that both these methods are efficient in inducing G0 state in BMSC and led to enhanced self-renewal ability, osteoblastic differentiation and ectopic bone formation upon transplantation in vivo. These changes were associated with significant changes in global gene expression inducting upregulation of intracellular signaling pathways known regulate osteoblast differentiation and bone formation. We conclude that induction of quiescence in cultured BMSC provides a useful model for analysis of mechanisms that might be relevant to the biology of BMSC in vivo.

2. Materials and methods

2.1. Cell culture

Both human and mouse cells were employed. The hBMSC-TERT cell line which is a proven model for primary hBMSC due its stable phenotype (Simonsen et al., 2002; Twine et al., 2018) was used for transcriptome analysis. Primary human and mouse BMSC were used to corroborate the results obtained in hBMSC-TERT cells (Supplementary Table 1).

Human bone marrow stromal stem cells (hBMSC) (from healthy donors) were purchased from Texas A&M Health and Science Centre (Texas, USA) and used between passages 1–3. hBMSC-TERT were derived in Prof. Moustapha Kassem's laboratory by overexpressing human TERT (*telomerase reverse transcriptase*) gene in hBMSC (Simonsen et al., 2002). Mouse bone marrow stromal stem cells (mBMSC) were isolated from 8-week old C57BL/6 mouse hind limb bones as described previous (Soleimani and Nadri, 2009). BMSC were maintained as asynchronous proliferating cultures (pre-G0) in growth medium (MEM-alpha, 10% fetal bovine serum (FBS), penstrep, glutamax) (Gibco) and passaged at 60–70% confluency. 2% methylcellulose (MC) (Sigma) stock used for suspension culture was prepared as described previously (Arora et al., 2017). To induce G0 in MC suspension culture, asynchronously proliferating (pre-G0) BMSC were trypsinized and re-suspended in MC at a density of 10^6 cells per 10 ml of suspension medium and cultured for 48 h. Briefly, for preparing 10 ml MC suspension, 10^6 cells were collected in 500 µl of growth medium in 50 ml falcon, to which was added FBS (10%), penstrep, glutamax, HEPES (10 mM, pH 7.3) and 2% MC (up to 10 ml, final MC concentration 1.3%). For reactivation studies, MC cell suspension was diluted with 40 ml PBS (pre-warmed at 37 °C), centrifuged at 1800*g* for 30 mins at room temperature without brakes, the supernatant discarded and the loose pellet dispersed by gentle pipetting. Cells were washed twice with 40 ml of warm PBS, first at 800 g for 15 min, and then at 250 g for 5 min (Arora et al., 2017). This harvest procedure yielded optimal viability. The harvested cells were counted and checked for viability using trypan blue before replating on tissue culture plastic dishes or lysed directly for RNA and protein isolation.

Polyacrylamide gels (PAA) substrates of different stiffness were prepared by cross-linking 40% PAA and 2% bis-acrylamide solution (BioRad) mixed at different concentrations in PBS, as described previously (Pelham and Wang, 1997). Details of the substrate preparation and rigidity values for different combinations of PAA and bis-acrylamide were as described (Tse and Engler, 2010). Briefly, the gels were prepared between two parallel glass cover slips, one coated with 3-APTMS ((3-aminopropyl) trimethoxysilane) (Sigma) and the other with octadecyl-trichlorosilane (Sigma), to render the cover slip respectively adherent and non-adherent to gel. Following cross-linked and gelling, the non-adherent plate was removed, and the gel was coated with type I collagen (Advanced Biomatrix) using sulfo-SANPAH (sulfosuccinimidyl 6-(4′-azido-2′-nitrophenylamino) hexanoate) based conjugation (Yeung et al., 2005) under UV for 15 min. Gels were washed with PBS and maintained under 50 µg/ml of type I collagen solution at 4 °C overnight. The control cover slip was also coated with Type 1 collagen. To avoid any effect of support glass plate on cellular mechano-sensing (Buxboim et al., 2010), we prepared thick gels (~400 µm), by keeping the volume of the gel solution constant at 200 µl. To induce G0 on soft PAA gels, cells were seeded at low density (1000 cells/cm^2) to avoid any cell-cell mechano-signaling via matrix (Reinhart-King et al., 2008) and cultured for 48 h in growth medium. Before seeding cells, excess collagen was removed and the gel was equilibrated in growth medium for 1 h. For reactivation studies, G0-arrested BMSC were harvested by trypsinization from the gel, and replated on tissue culture plastic in growth medium or lysed directly for RNA and protein isolation.

2.2. Immunofluorescent staining

Ki67 staining: Cells were fixed in 4% buffered formalin and were incubated overnight with Ki67 (Dako, 1:100) at 4 °C. After, cells were washed and incubated with a fluorescent secondary (Alexa Fluor, Life Technologies, 1:1000) for 1 h at RT, before counterstaining with DAPI (Sigma).

Actin & vimentin staining: hBMSC were fixed with 4% paraformaldehyde (PFA), pH 7 in 1% Triton-X-100 (Sigma) (1:1) for 1 min on ice. Cells were then washed twice with cytoskeleton stabilizing buffer (CSB) (60 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)), 27 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)), 10 mM EGTA ((ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)), 4 mM magnesium sulphate (heptahydrate), pH 7), and fixed again with 4% PFA for 5 mins on ice. Cells were washed with CSB, blocked with 1.5% BSA in 0.5% Triton X-100 for 30 mins on ice and incubated with anti-vinculin antibody (Sigma, 1:400) overnight at 4 °C, detected with Alexa Fluor-568 Rabbit anti-mouse antibody (Thermo, 1:1000). Actin was detected with Alexa Fluor-488 Phalloidin (Thermo, 1:400) and nuclei counterstained with Hoechst 33342, prior to imaging on a laser scanning confocal microscope (LSM, Carl Zeiss).

BrdU staining: Cells were pulsed with 100 µM BrdU (Sigma) for 1 h at 37 °C, then processed for detection of incorporated BrdU using anti-BrdU antibody (DSHB, 1:100) as described (Dhawan and Helfman, 2004).

2.3. RNA isolation and qPCR

Cells were lysed in 1 ml of TRIzol (Invitrogen) and total RNA was isolated per the manufacturer's recommendations and as previously published (Cheedipudi et al., 2015). RNA was quantified on nanodrop spectrophotometer (Thermo Scientific), and cDNA prepared with 1 µg of total RNA using Invitrogen SSIII RT kit as per manufacturer's protocol. Real time PCR was performed on ABI HT7000 and fold change was calculated by $2^{-\Delta\Delta Ct}$ method, using GAPDH to normalize values. List of primers used is provided in Supplementary Table 2.

2.4. CFU-assay

Pre-G0 or G0 (MC culture) cells were used for CFU-f assay as previously described (Subramaniam et al., 2013). hBMSC (500 cells) or