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Research review paper

Skeletal stem cell isolation: A review on the state-of-the-art microfluidic label-free sorting techniques

Miguel Xavier [a,b], Richard O.C. Oreffo [b], Hywel Morgan [a,*]

[a] Faculty of Physical Sciences and Engineering, Institute for Life Sciences, University of Southampton, SO17 1BJ, United Kingdom.
[b] Centre for Human Development, Stem Cells and Regeneration, Institute of Developmental Sciences, Southampton General Hospital, Tremona Road, SO16 6YD Southampton, United Kingdom.

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ABSTRACT

Skeletal stem cells (SSC) are a sub-population of bone marrow stromal cells that reside in postnatal bone marrow with osteogenic, chondrogenic and adipogenic differentiation potential. SSCs reside only in the bone marrow and have organisational and regulatory functions in the bone marrow microenvironment and give rise to the haematopoiesis-supportive stroma. Their differentiation capacity is restricted to skeletal lineages and therefore the term SSC should be clearly distinguished from mesenchymal stem cells which are reported to exist in extra-skeletal tissues and, critically, do not contribute to skeletal development.

SSCs are responsible for the unique regeneration capacity of bone and offer unlimited potential for application in bone regenerative therapies. A current unmet challenge is the isolation of homogeneous populations of SSCs, *in vitro*, with homogeneous regeneration and differentiation capacities. Challenges that limit SSC isolation include a) the scarcity of SSCs in bone marrow aspirates, estimated at between 1 in 10–100,000 mononuclear cells; b) the absence of specific markers and thus the phenotypic ambiguity of the SSC and c) the complexity of bone marrow tissue.

Microfluidics provides innovative approaches for cell separation based on bio-physical features of single cells. Here we review the physical principles underlying label-free microfluidic sorting techniques and review their capacity for stem cell selection/sorting from complex (heterogeneous) samples.

Contents

Abbreviations: SSC, skeletal stem cells; BM, bone marrow; MSCs, mesenchymal stem cells; BMSCs, bone marrow stromal cells; BMA, bone marrow aspirates; CFU-F, colony forming unit-fibroblastic; FACS/MACS, fluorescence/magnetic-activated cell sorting; CTCs, circulating tumour cells; DLD, deterministic lateral displacement; DEP, dielectrophoresis; GEDI, geometrically-enhanced differential immunocapture; WBC, white blood cells; RBC, red blood cells; QMS, quadrupole magnetic flow sorter; HSC, haematopoietic stem cells; PBPC, peripheral blood progenitor cell; FFF, field-flow fractionation; RT-DC, real-time deformability cytometry.

* Corresponding author at: Office 2105, Building 85, Institute for Life Sciences, Highfield Road, University of Southampton, SO17 1BJ, United Kingdom.
E-mail addresses: j.xavier@soton.ac.uk (M. Xavier), roco@soton.ac.uk (R.O.C. Oreffo), hm@esc.soton.ac.uk (H. Morgan).

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1. Introduction

Current estimates indicate that in developed countries the majority of children born after the millennium will live to witness their 100th birthday (Christensen et al., 2009). While this heralds a significant achievement for global health and medical care, such advances in longevity are typically accompanied with exacerbated health problems and increased demands for personalised, directed and effective regenerative therapies (Christensen et al., 2009; Rachner et al., 2011). Within the musculoskeletal arena, increased bone trauma and bone disease are associated with advancing years and stem cell-based therapies have been suggested as a possible approach to address these issues (Bianco, 2015; Dawson et al., 2014; Rachner et al., 2011).

Bone has unique capacity for regeneration, indicating the potential of a multipotent stem cell resident in the bone with the capacity to support bone tissue engineering, skeletal stem cell transplantation or pharmacological studies targeting bone disease (Bianco, 2015). Indeed, almost half a century ago, Friedenstein and colleagues, first documented the occurrence of osteogenesis in heterotopic transplants of bone marrow (BM) stromal cells *in vivo*, providing evidence of a stem cell with the capacity to generate bone (Friedenstein et al., 1966, 1968). BM stroma-derived cell populations with replicative and differentiation capabilities are typically referred to as mesenchymal stem cells (MSCs). However, this term has proved itself to represent a highly heterogeneous cell population when these cells are grown *in vitro*, comprising often several progenitor cells for different terminal cell lineages. The heterogeneous population of cultured plastic adherent cells isolated from the bone marrow, widely used in the community to study bone reparation, will be referred to as bone marrow stromal cells (BMSCs). The term skeletal stem cell (SSC), proposed by Friedenstein and Owen, is used in this review to refer specifically to the self-renewing stem cell of the bone marrow stroma responsible for the regenerative capacity inherent to bone. The SSC displays osteogenic, chondrogenic and adipogenic differentiation potential *in vivo* and the capacity to form a functional BM-haematopoietic microenvironment (Bianco, 2015; Bianco and Robey, 2015; Friedenstein et al., 1966, 1968; Friedenstein and Owen, 1988; Gothard et al., 2011).

Current SSC isolation and purification techniques usually rely on density centrifugation of BM aspirates (BMA) followed by culture adherence to tissue culture polystyrene. At clonal seeding densities, the formation of distinct colonies, named colony forming units-fibroblastic (CFU-F) can be observed, a fraction of which (approximately 15%), contains the SSC sub-population. The skeletal stem cell population is thought to be of pericyte origin and to reside *in vivo* over the BM sinusoids (Bianco, 2015; Gothard et al., 2011; Janeczek et al., 2015a). In theory, one single stem cell is sufficient for application in stem cell regenerative therapies due to their accepted replicative capacity. However, a key challenge hampering clinical translation is the necessity to enrich/obtain, *in vitro*, a sufficient population of cells with a homogeneous regeneration and differentiation capacity (Gothard et al., 2011; Poon et al., 2015). Critically, expansion *ex vivo* can contribute to an increase in cell heterogeneity linked to a loss of proliferative and differentiation capacity (Gothard et al., 2011). To date, researchers have struggled to develop isolation techniques that will provide SSC populations of sufficient high purity and cell yield without compromising cell viability.

Although a specific marker for the SSC remains elusive, positive-selection based on the application of one or more SSC-surface markers using fluorescence- or magnetic-activated cell sorting (FACS and MACS), is widely used. One commonly used approach is density centrifugation followed by MACS separation using positive-selection of Stro-1^{+} (a putative SSC marker with some 10–15% reactivity of BMSCs) cells and plastic adherence to enrich for SSCs (Gothard et al., 2013, 2014).

Limitations in SSC isolation techniques are related to a) the scarcity of SSCs in BMA (1 in 10–100,000 mononuclear cells) (Jones and McGonagle, 2008), b) the absence of specific markers and thus the phenotypic ambiguity of the SSCs (Bianco et al., 2013; Gothard et al., 2011, 2014; Tare et al., 2008) and, c) the complexity of bone marrow tissue with cell types displaying overlapping features to the SSCs (Fawcett and Bloom, 1994; Junqueira and Carneiro, 2005). To address some of these issues, microfluidic technologies offer new routes for single cell analysis and high throughput cell sorting that do not rely on cell-surface markers but rather on distinct cell phenotypic features. This review summarises recent advances in microfluidic sorting techniques and their potential applicability to the isolation of pure and homogeneous SSC populations for academic and clinical applications.

2. State-of-the-art cell isolation techniques and their caveats for skeletal stem cell sorting

The main approaches employed in SSC sorting are fluorescence and magnetic cell sorting combined with plastic culture adherence (Tare et al., 2008) Herzenberg and his colleagues first described fluorescent activated cell sorting (FACS) in 1972 (Bonner et al., 1972), while Miltenyi Biotec developed magnetic sorting and registered the trademark MACS (Miltenyi et al., 1990). Both depend on the use of antibodies to specific antigens that are either present on the cell membrane, or in the cell cytoplasm or even nucleus. Cell sorting is performed using either positive or negative selection depending on whether the cells targeted by the antibodies are those of interest or the contaminants. The principle of separation differs depending on the method employed. In FACS, cells are suspended in droplets and sorted depending on the presence or absence of a fluorescent tag. MACS uses magnetic beads attached to a primary antibody allowing tagged cells to be retained within a flow-through device by a strong magnetic field (Plouffe et al., 2015).

FACS can process thousands of cells per second in a serial manner, and provides the possibility of selection based on targeting intracellular components, multiple antigens, or antigen density. MACS is a bulk cell sorting technology and is fluorescence-independent. In theory, both FACS and MACS should be able to isolate 100% pure cell populations if appropriate cell specific selection markers are available. However, cost (reagents, antibodies, flow cytometer), time (typically 7–8 h protocols with possible loss of cell viability as a consequence) and the need for trained personnel are considerable limitations for both techniques (Karabacak et al., 2014; Plouffe et al., 2015). Nevertheless, the major hurdle in the application of FACS or MACS for SSC sorting is the lack of a specific cell marker for the SSC. For example, the widely used antibody Stro-1 reacts with approximately 10–15% of BMSCs and provides only enrichment rather than selection of SSCs.

Stro-1 was first identified by Simmons and Torok-Storb in 1991 and is a relatively widely used marker for SSC sorting and analysis, and for selection of high growth-potential CFU-Fs (Kolf et al., 2007; Lin et al., 2011; Simmons and Torok-Storb, 1991; Tare et al., 2008). In 2003, Gronthos et al. obtained a 950-fold enrichment of CFU-Fs by MACS separation of Stro-1^{bright} human bone marrow stromal cells (Gronthos et

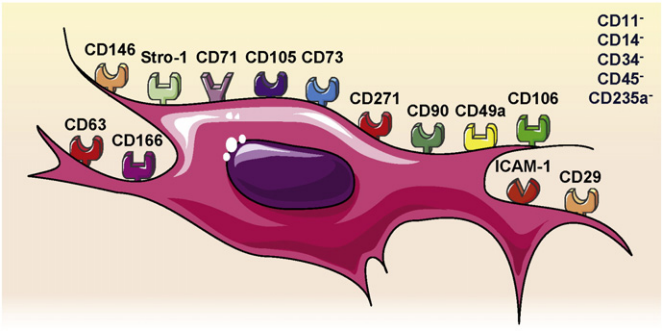

Fig. 1. – Surface marker expression. Cell surface antigens of human bone marrow skeletal stem cells.