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# *Ex vivo* expansion of rat bone marrow mesenchymal stromal cells on microcarrier beads in spin culture

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**Abstract**

Bone marrow mesenchymal stromal cells (BM-MSC) are attractive candidates for connective tissue regeneration. Currently, their use is limited by poor overall cell survival and high apoptosis rates upon transplantation *in vivo*. We hypothesized that disruption of cell–extracellular matrix contact either during cell expansion or immediately prior to cell transplantation may impair cell viability and facilitate apoptosis. We therefore investigated whether BM-MSC can be expanded on microcarrier beads in spin culture and directly transplanted. This novel approach removes the need for the repeated trypsinizations that are usually required for expansion and transplantation. CultiSpher-S gelatin microcarrier beads supported Fisher and transgenic green fluorescent protein $(GFP)^+$ Sprague Dawley rat BM-MSC expansion. Bead-expanded BM-MSC could still be differentiated along the chondrogenic, osteogenic and adipogenic lineages. In the short term, direct subcutaneous transplantation of cells expanded on microcarriers was associated with significantly less apoptosis than trypsinized control cells. In the long term, BM-MSC expanded on microcarrier beads induced *de novo* trabecular bone formation *in vivo*. This novel approach present several advantages over current expansion–transplantation protocols for mesenchymal tissue regeneration.


*Keywords:* Mesenchymal stromal cell; Gelatin microcarrier; Bone healing; Apoptosis; Transplantation; Cell culture

## 1. Introduction

Adult mesenchymal stromal cells (MSC) have been proposed as attractive candidates for regenerative therapy of connective tissues. Progenitors within this population give rise to bone, fat and cartilage, but differentiation to tendon, skeletal and cardiac muscle has also been reported [1–3]. MSC are thought to reside in most tissues and organs [4], but the most common source of these cells is the bone marrow (BM-MSC) [5]. While phenotypic markers have been defined for cultured BM-MSC (reviewed in [6]), their usefulness in prospectively purifying these cells has not been proven. To date, the isolation of MSCs, either from bone marrow or other sources, is based primarily on their adhesion to plastic, their expansion potential and ultimately their differentiation along multiple mesenchymal lineages (reviewed in [7]).

One of the problems limiting the use of MSCs for regenerative approaches arises from the fact that their extended expansion in culture leads to loss of multipotentiality and senescence [8–10]. However, growth factors such as bFGF have the potential to improve the maintenance of these progenitors *in vitro* [11]. In addition, another critical factor in the maintenance of an immature phenotype during expansion is likely to be the availability of appropriate extracellular substrates to which the cells can adhere. Intracellular signals triggered by the interaction of adhesion molecules with extracellular matrix

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components are critical for cell survival both *in vivo* and *in vitro*, and their interruption leads to a specialized form of apoptosis called anoikis [12]. However, during *in vitro* expansion the interactions established with these substrates are cyclically interrupted every time the cells are trypsinized and replated, potentially reducing viability and limiting our ability to expand difficult populations. More importantly, these cell–matrix interactions are disrupted when the cells are finally detached from tissue culture plates just prior to their transfer *in vivo*. This could contribute to the dismal overall viability and high apoptosis rates observed upon transplantation of unmodified BM-MSC.

Here, we tested the use of microcarrier beads in suspension culture as an alternative approach to expanding BM-MSC. This approach allows the expansion of BM-MSC populations without trypsinization. Furthermore, microcarrier beads "loaded" with cells can be directly transplanted *in vivo*, dispensing with the requirement to detach the cells from their substrate.

Microcarrier suspension culture has been extensively used to expand anchorage-dependent cells in culture. Surface area, bead size, cell adhesion and cell spreading are all critical factors that may modulate cell attachment and ultimately their expansion potential [13,14]. Among stem/progenitor cells, mouse embryonic stem cells have been successfully expanded on glass microcarriers in suspension culture [15]. However, microcarrier used to support BM-MSC culturing, viability during expansion and transplantation as well as their potential use to induce bone formation has not been examined. Therefore, we selected three unique microcarriers and examined their potential to support BM-MSC adhesion and expansion. First, CultiSpher-S is a macroporous, highly cross-linked gelatin microcarrier that is enzymatically degradable and supports chondrogenic progenitor expansion and differentiation [16,17]. Autologous keratinocytes expanded on similar gelatin microcarriers and directly transplanted to non-healing leg ulcers did significantly improve wound healing [18]. Second, Cytopore-2$^{TM}$ is a macroporous, non-toxic and biodegradable cellulose microcarrier bead that has been extensively used for long-term culture of recombinant CHO cell lines [19,20]. Lastly, the Cytodex-1$^{TM}$ microcarrier is a cross-linked dextran matrix that has also been used to culture a variety of cell types [21,22].

This study examined whether these microcarriers can be used to effectively expand wild type Fisher 344 rat BM-MSC and transgenic green fluorescent protein (GFP)$^{+}$ Sprague Dawley (SD) rat BM-MSC populations. To support the notion that immature subpopulations are retained, we examined whether microcarrier-expanded qcells could differentiate along chondrogenic, osteogenic and adipogenic lineages. In short-term experiments, we examined if direct *in vivo* subcutaneous transplantation of BM-MSC on microcarriers underwent less apoptosis. Finally, in long-term experiments we examined *in vivo de novo* trabecular bone in experimentally generated bone defects.

## 2. Materials and methods

### 2.1. Rat BM-MSC cell spin and plate culture

Bone marrow isolated from 8-week male Fisher 344 rats (Charles Rivers, USA) and GFP$^{+}$ transgenic SD rats (SLC, Japan) were used for BM-MSC isolation. Long and pelvic bones dissected from euthanized rats were crushed to release total bone marrow cells as described previously [23]. All procedures were approved by The University of British Columbia, Animal Care Committee.

CultiSpher-S (diameter 130–380 μm) (Percell Biolytica, Astorp, Sweden), Cytodex-1$^{TM}$ (diameter 131–220 μm) and Cytopore-2$^{TM}$ (diameter 200–270 μm) (Amersham Biosciences, Picataway, New Jersey) were three microcarrier beads prepared for cell culture. Microcarriers were rehydrated in PBS, autoclaved, washed and soaked in 5 ng/mL fibronectin (Sigma, St Louis, MO, USA) overnight at 4 °C, washed and placed in spin culture flasks (Wheaton, Millville, NJ, USA) with MesenCult stem cell media, 15% serum (StemCell Technologies, Vancouver, BC, Canada) and 2.5 ng/mL human recombinant FGF-2 (Upstate, Lake Placid, NY, USA). Basic FGF was added to expand cells and maintain multipotentiality [11]. Total bone marrow cells ($1.5 \times 10^7$) were added to microcarrier (50 mg) suspension cultures or $2 \times 10^5$ cells/cm$^2$ seeded on conventional tissue culture plastic plates coated with 5 ng/mL fibronectin. During the first 24 h, no cell agitation was done in the microcarrier with cell suspension but this was followed with continuous stirring at 15–50 rpm on a Thermolyne Cellgro$^{TM}$ Stirrer (Krackeler Scientific Inc., Albany, NY, USA). The first media change occurred on day 5 and subsequently every 3 days.

Cell growth kinetics was monitored using a MTT proliferation assay kit (Promega, Madison, WI, USA) at selected time points. Ten to 100 μL aliquots of beads plus cells were placed in a 96-well plate, 20 μL mixture of MTT reagent was added and absorbance (490 nm) recorded at 4 h. For experiments comparing proliferation of parallel culture on tissue culture plastic, cells were plated into 96-well plates at an equivalent density to large plate cultures and proliferation assessed using the MTT assay. As rodent bone marrow isolates contain adherent macrophages that can expand in culture, we collected days 21 and 28 bead samples, embedded them in OCT (Sakura Finetek, Torrance, CA, USA) and prepared frozen sections that were DAPI (Molecular Probes, Eugene, USA) and CD45 or CD11b immunostained (BD Pharmingen$^{TM}$, Mississauga, Canada).

### 2.2. Scanning electron microscopy (SEM)

Suspension cell culture was sampled at selected time points and processed for SEM. Cells were fixed with 2.5% glutaraldehyde followed by post-fix with 2% osmium tetroxide and 1% tannic acid. After dehydration, cells were critical point dried and mounted for gold coating (with a Hummer VI Sputter Coater, Technics Inc., Alexandria, VA, USA). We examined approximately 200 microcarrier beads/group and SEM images were captured using a Cambridge Stereoscan 260 microscope (Cambridge, UK).

### 2.3. Rat BM-MSC differentiation

Differentiation potential of BM-MSC along bone, fat and cartilage lineages was assayed on cells expanded for 28 days on tissue culture plastic and bead cultures. Induction of osteogenesis [24], adipogenesis [25] and chondrogenesis [26] followed established protocols and all differentiation induction assays were carried out for 3 weeks.

### 2.4. RT-PCR and quantitative RT-PCR (qRT-PCR)

Regardless of culture approaches, cells were lysed by TRIZOL reagent (Invitrogen, Burlington, Ont., Canada). Total RNA was purified by chloroform extraction and isopropanol purification and treated with DNase I to remove possible contaminating DNA. Five hundred