The isolation and characterization of putative mesenchymal stem cells from the spiny mouse

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Abstract The bone marrow represents the most common source from which to isolate mesenchymal stem cells (MSCs). MSCs are capable of differentiating into tissues of the three primary lineages and have the potential to enhance repair in damaged organs through the principals of regenerative medicine. Given the ease with which MSCs may be isolated from different species the aim of this study was to isolate and characterize putative bone marrow derived MSCs from the spiny mouse, *Acomys cahirinus*. MSCs were isolated from the spiny mouse in a traditional manner, and based on plastic adherence, morphology, colony forming unit-fibroblast assays and functional assessment (adipogenic, osteogenic and chondrogenic differentiation potential) a population of putative mesenchymal stem cells from the compact bone of the spiny mouse have been isolated and characterized. Such methodological approaches overcome the lack of species-specific antibodies for the spiny mouse and could be employed for other species

where the cost of generating species-specific antibodies is not warranted.

Keywords Compact bone · *Acomys cahirinus* · Colony forming unit

Introduction

The bone marrow represents the most common source from which to isolate mesenchymal stem cells (MSCs) (Tang et al. 2006), however other sources include the Wharton's jelly of the umbilical cord (Fu et al. 2006), amniotic fluid (In 't Anker et al. 2003) and adipose tissue (Yanez et al. 2006). MSCs are relatively easy to harvest, can be readily expanded in culture and, in addition to being isolated from a number of tissues, have also been isolated from a number of species including conventional rodents (Zangi et al. 2006; Sung et al. 2008), cows (Bosnakovski et al. 2006) and horses (Vidal et al. 2006). Typically, MSCs are isolated on the basis of plastic adherence, negative selection for a range of cell surface markers and multi-lineage differentiation potential. MSCs isolated from different tissues and species have reproducible attributes, not only between isolates but also between laboratories.

MSCs are compatible with principles of regenerative medicine and have the potential to enhance repair in vital organs and tissues. In addition to their regenerative abilities, bone marrow derived-MSCs

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are immune-privileged, are effective in inducing tolerance and interact with T cells and other cell of the immune system (Le Blanc 2003; Pittenger and Martin 2004). Given the ease with which MSCs may be isolated from different species, the aim of this study was to isolate and characterize putative bone marrow derived MSCs from the spiny mouse, *Acomys cahirinus*.

The spiny mouse is a rodent species, native to regions of Egypt and Israel that we, and others, believe to be a more appropriate animal model for the study of fetal and neonatal development; owing to its precocial mode of development (Dickinson et al. 2008; Dickinson and Walker 2007; Dickinson et al. 2005; Ireland et al. 2008; Lamers et al. 1985). We have developed a number of models of neonatal/adult insufficiency (Dickinson et al. 2007; Ireland et al. 2009), following an insult during pregnancy and the isolation and characterization of MSCs that may be of benefit in regenerating the resulting deficient organs is of great interest to us.

Previous work in the spiny mouse has found that this species is more closely related to the gerbil, genus *Meriones*, than to the common mouse, genus *Mus* (Chevret et al. 1993). Our previous work has found that there is limited genuine cross-reactivity between mouse monoclonal antibodies and spiny mouse antigens (Dickinson H, unpublished observations). For this reason, putative MSCs were isolated from the spiny mouse in a traditional manner, based on plastic adherence, morphology, colony forming unit-fibroblast (CFU-F) assays and functional assessment, based on adipogenic, osteogenic and chondrogenic differentiation potential.

Methods

Animals

The spiny mice used in this study were housed and maintained as previously described (Dickinson and Walker 2007). Briefly, spiny mice are housed in a conventional animal facility and are routinely checked for pathogens. At the time of the described experiment, the colony of spiny mice was deemed to be virus and pathogen free (Serology and molecular diagnostics report October 2009, Institute of Medical and Veterinary Science, South Adelaide, Australia).

Tissue collection

The procedure for isolating putative MSCs from the spiny mouse (*A. cahirinus*) was adapted from a previously described method for isolating murine MPCs from the compact bone (Guo et al. 2006). Adult male spiny mice (30 ± 2 weeks of age; $n = 5$) were killed by cervical dislocation and the iliac crests were removed *en bloc* with the femurs and tibias.

Bone isolation

Under sterile conditions, the femur was separated from the tibia and iliac crest for each leg and the foot removed from the tibia and discarded. The iliac crests, femurs and tibias were then scraped free of all visible flesh using a scalpel. The bone marrow was flushed with Dulbecco's Modified Eagle Medium (DMEM; high glucose, sodium pyruvate, no L-Glutamine; Gibco, USA) supplemented with 10% Fetal Calf Serum (FCS) and 1% (v:v) Penicillin–Streptomycin. A single cell suspension was filtered through a $40 \mu\text{m}$ cell strainer, a live cell number determined and the cells discarded.

Once flushed of bone marrow, the iliac crests, femurs and tibias were crushed using a mortar and pestle in 5 mL of 3 mg/mL Collagenase type I (Invitrogen, USA) which was prepared in PBS with 2% FCS. The crushed bone solution was incubated with agitation at 37°C for 45 min, filtered through a $40 \mu\text{m}$ cell strainer and a live cell number determined.

Flow cytometric analysis

Cells isolated from the compact bone were analysed by flow cytometry for their expression of the murine MSC markers Sca-1, CD44, CD31 and CD45 (Table 1; all markers were purchased from BD Biosciences Pharmingen). Three samples were prepared for each marker of interest and included, (a) an unstained sample, (b) an isotype control, and (c) a cell preparation stained for the marker of interest. Cells isolated from the compact bone were counted, aliquoted at 1×10^6 cells/tube, with PBS and 2% FCS, centrifuged and the supernatant aspirated. Each cell aliquot was stained with either the antibody for the marker of interest or the corresponding isotype control, at a 1:400 dilution, and incubated at room temperature in the dark for 30 min. Following incubation, the cells were washed, centrifuged, the washing solution was aspirated and the cells were

Table 1 MSC antibodies

	MSC marker	Antibody	Isotype control	Conjugate
<i>PE</i> phycoerythrin, <i>FITC</i> fluorescein isothiocyanate	CD44	Anti-mouse CD44	Mouse IgG2a κ	PE
	CD31	Anti-mouse CD31	Mouse IgG2a κ	PE
	CD45	Anti-mouse CD45	Mouse IgG2b κ	FITC
	Sca-1	Anti-mouse Ly- $\hat{A}$ /E (Sca-1)	Mouse IgG2a κ	PE

resuspended in PBS with 2% FCS for analysis on an FC500 Flow Cytometer Analyser. The position of gates used to identify positively stained cells was set on the basis of the signal observed with the corresponding isotype control for the antibodies.

Tissue culture and colony forming unit assay

Isolated cells from the compact bone fraction ($n = 3$) were cultured at 37 °C in a humidified incubator with 5% (v:v) CO₂ and 95% (v:v) air infusion. Passage 0 cultures were plated in 25 cm² flasks and cultured in DMEM supplemented with 10% FCS and 1% (v:v) Penicillin–Streptomycin. After 48 h in culture, non-adherent cells were removed by aspirating and replacing the culture medium, which was changed every 4 days thereafter. At approximately 85% confluency, the cells were harvested and passaged into 75 cm² flasks. At the next passage, the cells were replated into 175 cm² flasks. At each passage, the numbers of colony forming units were counted. For a cluster of cells to be considered as a colony and to be included in the colony forming unit count, it contained a minimum of 50 cells, as defined by Friedenstein et al. (1974). These assays were used to determine the incidence of putative MSCs in the isolated compact bone fraction.

General growth characteristics and multi-lineage differentiation potential

A defining property of MSCs is their ability to undergo adipogenic, osteogenic and chondrogenic differentiation (Horwitz et al. 2005; Alison and Islam 2009). As a part of characterizing the putative spiny mouse MSCs, it was therefore necessary to assess their tri-lineage differentiation potential.

Adipogenesis and osteogenesis

Passage 5 cells were plated at a density of 50,000 cells/chamber in each chamber of a four well chamber slide

(Becton–Dickinson, USA). The cells were incubated for 24 h in DMEM containing 10% FCS and 1% (v:v) Penicillin–Streptomycin to enable adherence to the growth surface prior to the addition of adipogenic differentiation medium. To induce adipogenesis, the medium for two of the four chambers was replaced with adipogenic differentiation medium, which consisted of high glucose DMEM supplemented with 10% FCS, 1% (v:v) Penicillin–Streptomycin, 1 μ M dexamethasone, 60 μ M indomethacin, 5 μ g/mL insulin and 0.5 mM IBMX. To induce osteogenesis, the medium for two of the four chambers was replaced with osteogenic differentiation medium, which consisted of high glucose DMEM supplemented with 10% FCS, 1% (v:v) Penicillin–Streptomycin, 100 nM dexamethasone, 10 mM β -glycerophosphate and 0.05 mM ascorbic acid. The remaining two chambers from each plate that did not receive adipogenic/osteogenic medium were used as control wells. All medium were changed twice a week for three weeks. After 21 days of culture, the medium was aspirated, the cells were rinsed and then 35% ethanol was added to each well to fix the cells which were then stained with Oil Red O for visualization of lipid vacuoles in the cytoplasm or silver nitrate using the von Kossa procedure for detection of calcium deposition in osteocytes.

Chondrogenesis

For chondrogenesis, a micromass pellet culture system was used (Hegewald et al. 2004). Approximately 1.25×10^5 cells in 10 μ L of DMEM containing 10% FCS and 1% (v:v) Penicillin–Streptomycin were plated in the centre of each well of a four well culture dish (Becton–Dickinson, USA) and incubated at 37 °C in a humidified incubator with 5% (v:v) CO₂ and 95% (v:v) air infusion for 2 h to allow cell adherence. The chondrogenic differentiation medium, which consisted of high glucose DMEM supplemented with 10% FCS, 1% (v:v) Penicillin–

Streptomycin, 5 ng/mL TGF- β 1, 100 nM dexamethasone, 50 μ g/mL ascorbic acid, 40 μ g/mL proline, 100 μ g/mL pyruvate and 1% insulin/transferrin/selenium (ITS) medium supplement, was then carefully added to avoid disturbing the formed cell pellets to two of the four wells in the culture dish. The remaining two wells, which did not receive chondrogenic medium,

were used as control wells. Medium was changed twice a week for the first 7 days, after which it was changed every day for 2 weeks. After 3 weeks in culture, the medium was aspirated, the cells/pellets were rinsed with PBS and then 35% ethanol was added to each well to fix the cells/pellets. After fixation, the pellets were processed for paraffin embedding,

Fig. 1 Flow cytometric analysis of cells isolated from the compact bone of the spiny mouse for expression of Sca-1, CD31, CD44 and CD45. Cells isolated from the compact bone of the spiny mouse had negligible expression of Sca-1 (b), CD31 (e) and CD45 (k), whereas CD44 (h) was detected. This is

compared to the cells isolated from the compact bone of the positive control, C57BL/6 mice, which had expression for all markers analysed (c, f, i and l). There was negligible expression for the isotype controls (a, d, g and j) indicating minimal non-specific binding by the antibodies

sectioned at 5 μm thickness and then stained with alcian blue for detection of cartilage proteoglycan.

Data presentation

All results are presented as mean $\pm$ SEM. A two-way analysis of variance (ANOVA) was used to determine differences in the number of fibroblastic and non-fibroblastic colony forming units. Statistical significance was tested to the 5% level ($p < 0.05$).

Results

Recovery of cells from the compact bone and bone marrow

The procedure for isolating putative mesenchymal stem cells from the spiny mouse, as used in this study, yielded a heterogeneous population of cells, which recovered a total of $8.1 \times 10^7 \pm 1.4 \times 10^7$ cells from the compact bone and a total of $1.7 \times 10^8 \pm 4.0 \times 10^7$ cells from the bone marrow.

Flow cytometric analysis of compact bone isolates

The heterogeneous population of cells isolated from the compact bone of the spiny mouse contained a sub-population of cells that positively expressed CD44. There was negligible expression of Sca-1, CD31 and CD45 in the compact bone cell isolate (Fig. 1b, e, k and h). This was compared to cells isolated from the compact bone of C57BL/6 mice, which had a sub-population of cells that were positive for Sca-1, CD31, CD44 and CD45 (Fig. 1c, f, i and l).

Incidence of putative mesenchymal stem cells from compact bone

When cells isolated from the compact bone were plated at increasing densities, colonies of adherent fibroblast-like cells were visible following seven days in culture. A ‘fibroblastic colony’ was a colony consisting of cells which was long and spindle shaped (Fig. 2b), and was generally substantially larger than a ‘non-fibroblast colony’, which consisted of cells that were of a ‘cobblestone’, epithelial-like morphology (Fig. 2c,

Fig. 2 Colony forming unit (CFU) assay—the incidence of putative MSCs in the compact bone of the spiny mouse. The number of CFU colonies arising from the compact bone cell isolate of the spiny mouse when plated at increasing cell densities (**a**); two-way ANOVA $p < 0.0001$, with significant differences between groups indicated by *** ($p < 0.001$). A

‘fibroblastic colony’ consisted of cells that were long and spindle shaped (**b**). ‘Fibroblastic colonies’ were generally larger than ‘non-fibroblastic colonies’, which consisted of cells that had a more ‘cobblestone’, epithelial-like appearance (**c** and **d**). Scale bar represents 50 μm ; objective $\times 40$. NB: images were taken of colonies growing in the same culture flask

Fig. 3 Multilineage differentiation of fibroblast-like cells, putative spiny mouse MSCs isolated from the compact bone. After 21 days of culturing fibroblast-like cells in adipogenic differentiation medium, the formation of adipocytes was demonstrated by the presence of lipid droplets in the cytoplasm of differentiated cells, confirmed by oil red O staining (**a**). Similarly, culture of fibroblast-like cells in osteogenic differentiation medium for 21 days yielded an osteoblast phenotype, as demonstrated by mineral deposition following the von Kossa staining procedure (**b**). The formation of non-adherent solid macroscopic pellets, following culture of fibroblast-like cells in chondrogenic differentiation medium, was indicative of the presence of chondrocytes, and was confirmed by alcian blue staining for cartilage proteoglycans (**c**). Scale bar represents 50 μ m; objective $\times 40$

d). The colony forming unit assays resulted in approximately 10 fibroblast colonies per 1×10^6 cells plated, demonstrating that the incidence of putative mesenchymal stem cells isolated from the compact bone fraction was approximately 0.00001% (Fig. 2a).

General growth characteristics and multi-lineage differentiation potential

The fibroblast-like cells, those that constituted the ‘fibroblastic colonies’, were plastic adherent cells with a spindle-shaped fibroblast-like morphology and a population doubling time of approximately 20 h. When cultured in adipogenic differentiation medium, the morphology of the fibroblast-like cells undergoing adipogenesis began to change after approximately one week. Cells began to lose their fibroblastic, spindle shape, becoming more rounded with vacuoles in the cytoplasm, which were abundant after 21 days in culture. Following Oil red O staining, lipid droplets were visible in the cytoplasm (Fig. 3a). Control cells, fibroblast-like cells not cultured in adipogenic differentiation medium, maintained fibroblast-like morphology and showed no evidence of oil droplets in the cytoplasm (not shown). By contrast, no morphological changes were observed in fibroblast-like cells when cultured in osteogenic differentiation medium, however following 21 days in culture, mineral deposition was evident in fibroblast-like cells following staining with von Kossa (Fig. 3b). This was compared to control cells, fibroblast-like cells not cultured in osteogenic differentiation medium, which did not show mineral deposition following staining with von Kossa (not shown). Fibroblast-like cells, when cultured as a micromass pellet culture system in chondrogenic differentiation medium,

formed non-adherent solid macroscopic pellets within 7 days of culture, which continued to increase in size up to day 21, and stained positively with alcian blue, thus indicating the presence of cartilage-specific proteoglycans (Fig. 3c). Control cells, fibroblast-like cells cultured as a micromass pellet culture system with standard culture medium instead of chondrogenic differentiation medium, did not form macroscopic pellets but became a

dense confluent monolayer, which did not stain positively for alcian blue (not shown).

Discussion

MSCs have been isolated and characterized from a number of species, however they have not previously been isolated from the spiny mouse, *A. cahirinus*. The studies described here aimed to isolate and characterize putative MSCs from the spiny mouse, *A. cahirinus*, using traditional characterization methodology. Hence, on the basis of plastic adherence, morphology, fibroblastic colony forming unit assays and multilineage differentiation potential, a population of putative MSCs from the compact bone of the spiny mouse was isolated and characterized.

Stem cells have not previously been sourced from the spiny mouse; therefore this study represents the first isolation and characterization of these cells in this species. The spiny mouse is a desert-adapted rodent species and is of great interest to researchers, primarily because of its small litter size and precocial offspring. Its use as a research tool has increased over the past 10 years, particularly in the field of fetal and neonatal medicine, a field now focused on the potential use of stem cell therapies to repair/regenerate damaged tissues of the fetus and/or neonate. The isolation and characterization of MSC's from the spiny mouse provides the necessary first step for the continued use of the spiny mouse as a research tool in this discipline.

The numbers of putative MSCs isolated from the spiny mice in this study are comparable to numbers of MSCs isolated from humans and the rhesus monkey (Izadpanah et al. 2005). They also exhibited characteristic morphological features of MSCs, such as plastic adherence and spindle-shaped fibroblastic morphology (Eslaminejad et al. 2006; Izadpanah et al. 2005; Lange et al. 2005; Tang et al. 2006). MSCs differentiate by default in vivo into cell lineages of mesenchymal tissues, including bone, cartilage, fat, muscle, and myelosupportive stroma (Kyriakou et al. 2006). Similarly, isolated MSCs can be directed to differentiate in vitro, by culture with lineage specific differentiation medium, to differentiate down the osteogenic, adipogenic and chondrogenic lineages (Gregory et al. 2005). The fibroblast-like cells isolated

from the spiny mice in this study exhibited multilineage differentiation potential with the capacity for adipogenic, chondrogenic and osteogenic differentiation, as demonstrated for MSCs isolated from other species (Bosnakovski et al. 2006; Horwitz et al. 2005; Izadpanah et al. 2005; Eslaminejad et al. 2006; Tuli et al. 2003).

As expected, the ability to characterise spiny mouse MSCs on the basis of cell surface markers used routinely for mouse, was not possible in the current study. Isolation of MSCs via FACS remains difficult for most species as MSCs lack specific and unique markers; hence a combination of positive and negative cell surface marker expression is required to isolate MSC populations (Baddoo et al. 2003; Nadri and Soleimani 2007; Tropel et al. 2004). The most common surface markers positively selected for murine MSCs are CD106, CD105, CD73, CD29, CD44 and Sca-1. These positive cell surface markers in conjunction with the absence of hematopoietic and endothelial markers CD45, CD11b and CD31 are now routinely used in mouse MSC characterization. The markers used in this study (CD44, Sca-1, CD31 and CD45) therefore, were selected on the basis that they represented key markers available for positive and negative selection of mouse MSCs. However, given that the degree of genuine cross-reactivity (and alternatively non-specific binding) between mouse antibodies to spiny mouse antigens was unknown, sorting cells by positive and negative selection could not be carried out. The homology between the mouse and spiny mouse is the focus of ongoing studies, with whole genome and transcriptome analysis underway to definitively classify the spiny mouse as mouse/gerbil/other, and then develop species-specific reagents for further characterisation studies.

On the basis of their adherence to plastic, fibroblast colony formation, and adipogenic, osteogenic and chondrogenic differentiation potential, a population of putative MSCs from the compact bone of the spiny mouse has been isolated and characterized. The methodological approach in this study may be replicated for other species where species-specific cell surface markers are not available.

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