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Gas chromatography–mass spectrometry analysis of human mesenchymal stem cell metabolism during proliferation and osteogenic differentiation under different oxygen tensions

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ABSTRACT

Bone marrow derived human mesenchymal stem cells (hMSC) are the primary cell type in bone tissue engineering, and their life span during osteogenic differentiation is associated with changes in oxygen tension. As a ubiquitous regulator of cellular metabolic activity, oxygen tension influences the profiles of metabolites in the entire metabolic network and plays an important role in hMSC survival, function, and osteogenic differentiation. In the current study, we hypothesize that hMSC have a metabolic phenotype that supports growth in low oxygen environments and that this phenotype changes upon differentiation, leading to differential responses to oxygen tension. We developed a gas chromatography–mass spectrometry (GC–MS) based metabolic profiling approach to analyze the metabolic fate of ^{13}C-glucose in glycolysis and the tricarboxylic acid cycle (TCA) in undifferentiated hMSC and hMSC-derived osteoblasts (hMSC-OS) in response to perturbation in oxygen tension; specifically we compared changes induced by culture under 20% *vs.* 2% O_2. The isotope enrichments in the metabolites were calculated and used to infer activities of specific metabolic enzymes and the associated pathways. The results revealed contrasting metabolic profiles for hMSC and the hMSC-OS in both 20% and 2% O_2 states, with the most significant differences involving coupling of glycolysis to the TCA cycle, glutaminolysis, and the malate-aspartate shuttle. The results have important implications in defining the optimal culture conditions for hMSC expansion and osteogenic differentiation.

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1. Introduction

Human mesenchymal stem cells (hMSCs) isolated from bone marrow and other tissue sources are an important cell source in bone tissue engineering due to their capacity to be expanded in culture and to undergo osteogenic differentiation upon induction. Among hMSCs isolated from multiple tissue sources, bone marrow-derived hMSCs have been most extensively characterized and tested in bone tissue regeneration (Robey, 2011; Szpalski et al., 2012). Despite the effort, the osteogenic ability of the hMSC-based constructs remains inferior to that of autologous bone grafts, the benchmark for bone repair. A significant challenge for the engineered constructs is to maintain the viability and function of the transplanted hMSC in scaffolds; loss of over 70% of MSCs in the transplanted constructs in rodent models within weeks of implantation has been reported (Giannoni et al., 2010; Jäger et al., 2007). MSCs undergo very distinct phenotypic changes as they differentiate from primitive bone marrow stromal cells into osteoblasts that are responsible for bone matrix formation and its subsequent mineralization (Clarke, 2008). These phenotypic changes should also be accompanied by significant changes in their metabolic and energy demands. In addition, transplantation of engineered bone constructs is often accompanied by drastic changes from a nutrient-rich *in vitro* culture environment to an injury site with limited nutrient and oxygen supply prior to the establishment of vasculature. Thus, understanding the changes in the hMSC metabolic phenotype as they differentiate into osteoblasts is important in hMSC's application in bone tissue engineering (Giannoni et al., 2010; Utting et al., 2006).

In the stroma of the bone marrow, hMSCs reside in a hypoxic environment where oxygen tension is as low as 2% (Ma et al., 2009). *In vitro* studies have shown that culture under 2–3% O_2 (referred to as hypoxia) stimulated hMSC proliferation and better

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preserved their clonogenicity and multi-potentiality (Grayson et al., 2007, 2006; Dos Santos et al., 2009; Estrada et al., 2012; Fehrer et al., 2007; Lavrentieva et al., 2010). Metabolically, hMSCs consume glucose and produce lactate, even under normoxic conditions, a phenomenon similar to the Warburg effect seen in tumor cells (Vander Heiden et al., 2009). They also exhibit sensitivity to electron transport inhibitors, suggesting a reliance on both aerobic glycolysis and oxidative-phosphorylation for ATP production (Pattappa et al., 2010). In contrast, osteoblasts are in close contact with the vasculature at an oxygen tension of 5–9% and have high mitochondrial activity and oxygen demand that is necessary for sustaining matrix secretion and mineralization (Nicolaije et al., 2012; Zahm et al., 2008). Understanding cellular responses to oxygen tension as hMSC differentiate into osteoblasts requires detailed analysis of their metabolic patterns.

The hypothesis of the current study is that hMSC osteogenic differentiation is associated with changes in their metabolic phenotype, leading to differential responses to changes in oxygen tension. As a ubiquitous regulator of cellular metabolic activity, oxygen tension influences the profiles of metabolites in the entire metabolic network. While measurement of extracellular metabolites has been commonly used and provided useful information, the approach has large variation and is restrained to pre-determined pathways. In the current study, gas chromatography–mass spectrometry (GC–MS) was used to profile metabolism of ^{13}C-labeled glucose in both hMSC and the hMSC-derived osteoblasts (hMSC-OS) at two different oxygen levels. The isotope enrichments in the metabolites were calculated and used to infer activities of specific metabolic enzymes and the associated pathways. The results revealed contrasting metabolic profiles for hMSC and the hMSC-derived osteoblasts in response to oxygen levels.

2. Materials and methods

2.1. hMSC culture and osteogenic differentiation

Frozen hMSCs at passage 1 in freezing media (1×10^6 cell/mL/vial in αMEM, 2 mM L-glutamine, 30% FBS, and 5% DMSO) were obtained from the Tulane Center for Gene Therapy and cultured following the method outlined in our prior publications (Zhao and Ma, 2005; Grayson et al., 2004). The hMSCs were isolated from the bone marrow of healthy donors ranging in age from 19 to 49 years based on plastic adherence, being negative for CD34, CD45, CD117 (all less than 2%) and positive for CD29, CD44, CD49c, CD90, CD105 and CD147 markers (all greater than 95%), and possessing tri-lineage differentiation potential upon induction *in vitro*. Briefly, hMSCs at passage 6 were expanded and cultured using α-MEM and 1% Penicillin/Streptomycin (Life Technologies, Carlsbad, CA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) (growth medium) in a standard CO_2 incubator (37 °C and 5% CO_2). For hypoxic culture, hMSC were seeded at 800 cells/cm^2 and cultured under 2% O_2, 5% CO_2, and balanced N_2 in a C-Chamber (BioSpherix, Lacona, NY). Media, preconditioned under the respective oxygen tension, was changed every other day. Cells from the two oxygen conditions were harvested by trypsinization and manually counted using a hemocytometer. hMSCs from multiple donors were used in the study and the results were reported in the main text and the Supplemental Materials. All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

For osteogenic induction, hMSCs at passage 5 were cultured for 21 days in a standard 5% CO_2 incubator in osteogenic media, which consisted of high glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin, 100 nM dexamethasone, 10 nM sodium-β-glycerophosphate, and 12.8 mg/L ascorbic acid-2 phosphate (osteogenic medium). The extent of osteogenic differentiation was confirmed by Alizarin Red staining before and after exposure to hypoxia.

Spent media samples were collected from hypoxic and normoxic cultures. Glucose and lactate concentrations were determined using a 2500 Biochemistry Select Analyzer (YSI Incorporated, Yellow Springs, OH). These values were used to determine glucose consumption (G) and lactate production (L) in cultures in order to calculate the L/G molar ratios.

2.2. ^{13}C-glucose labeling of hMSC and hMSC-OS

Incorporation of ^{13}C-glucose in the growth and osteogenic media was performed by supplementing the glucose-free versions of growth (Athena ES, Baltimore, MD) and osteogenic medium with a 2:2:1 mixture of unlabeled, U-^{13}C-, and $^{13}C_1$-labeled glucose (Cambridge Isotopes Laboratories, Andover, MA) at the same concentration as typically used in those media (1 g/L and 4.5 g/L, respectively). All other medium components were as provided in the commercial medium. Cells were cultured for three days at 20% O_2 (normoxic) or 2% O_2 (hypoxic) in unlabeled medium at which point the culture medium was replaced with isotope enriched medium and cultured for an additional three days continuing under the same oxygen tension the cells were in before. For the hMSC-OS, cells were cultured and differentiated in unlabeled medium supplemented with differentiation factors for 21 days at 20% O_2. The regular osteogenic induction media was replaced with the labeled osteogenic medium at day 21 and cultured in a standard CO_2 incubator (20% O_2) or in a C-chamber (BioSpherix, Lacona, NY) at 2% O_2 and 5% CO_2 for three days. Glutamine labeling was done using glutamine-free growth medium (Athena ES, Baltimore, MD) supplemented with a 40:60 mixture of unlabeled and U-^{13}C-labeled glutamine (Cambridge Isotopes Laboratories, Andover, MA) at the same concentration as typically used in these media. Cells were cultured for three days at 20% or 2% O_2 in unlabeled medium at which point the culture medium was replaced with isotope enriched medium and cultured for three more days.

2.3. Metabolite extraction and derivatization

Cells were harvested by removing the medium, quickly washed with PBS (<15 s) and then flash-frozen by adding liquid nitrogen directly into the culture dish to quench the metabolism. The plates were extracted with an acetonitrile:water (1:1) solution containing norleucine as an internal standard (0.085 mg/mL norleucine in the water phase). The acetonitrile:water solution was added to the culture plates, the cells were scraped off of the dishes, transferred to an Eppendorf tube, and the extract was centrifuged at 4000 rpm for 10 min at 4 °C. Insoluble material was pelleted and the clarified supernatant transferred to a clean, silanized 1 mL Reacti-Vial for derivatization. The extracts were dried under vacuum overnight and dissolved in 20 μL pyridine and 20 μL N-methyl-N-(*tert* butyldimethylsilyl) trifluoro-acetamide containing 1% *tert*-butyldimethylchlorosilane (Thermo Scientific, Rockford, IL). The reaction vial was sealed under argon, heated at 75 °C for 60 min (Mawhinney et al., 1986a) to effect derivatization, and then cooled to room temperature. Derivatized samples were analyzed by GC–MS within 24 h of derivatization. Studies with standard compounds show no change in the relative intensities of a wide range of metabolites over this period.

2.4. GC–MS analysis of metabolite extracts

Derivatized metabolite samples (1 μL) were injected in splitless mode into an HP Agilent 6890 series gas chromatograph coupled with an HP Agilent 5973 mass selective detector and separated on a 30 m DB-5 column (J&W Scientific, Folsom, CA) (Wittmann et al.,

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