Research Article

For reprint orders, please contact: reprints@futuremedicine.com

Regenerative Medicine

BMP2 repression and optimized culture conditions promote human bone marrow-derived mesenchymal stem cell isolation

Aim: Human mesenchymal stem cells (hMSC) are multipotent progenitor cells. We propose the optimization of hMSC isolation and recovery using the application of a controlled hypoxic environment. **Materials & Methods:** We evaluated oxygen, glucose and serum in the recovery of hMSC from bone marrow (BMhMSC). Colony forming units-fibroblastic, cell numbers, tri-lineage differentiation, immunofluorescence and microarray were used to confirm and characterize BMhMSC. **Results:** In an optimized (2% O_2, 4.5 g/l glucose and 5% serum) environment both colony forming units-fibroblastic ($p = 0.01$) and cell numbers ($p = 0.0001$) were enhanced over standard conditions. Transcriptional analysis identified differential expression of bone morphogenetic protein 2 (*BMP2*) and, putatively, chemokine (C-X-C motif) receptor 2 (*CXCR2*) signaling pathways. **Conclusion:** We have detailed a potential milestone in the process of refinement of the BMhMSC isolation process.

Keywords: BMP2 • CFU-F • hypoxia • mesenchymal stem cells • physiological normoxia • transcriptome

Alasdair Gawain Kay[‡,1], Tina Patricia Dale[‡,1], Khondoker Mehedi Akram[‡,1], Param Mohan[1], Karen Hampson[1], Nicola Maffulli[1,2], Monica A Spiteri[1,3], Alicia Jennifer El Haj[1] & Nicholas Robert Forsyth*[,1]

[1]Institute for Science & Technology in Medicine, Guy Hilton Research Centre, Keele University, Stoke-on-Trent, Staffordshire, ST4 7QB, UK

[2]Centre for Sport & Exercise Medicine, Queen Mary University of London, Barts & The London School of Medicine & Dentistry, Mile End Hospital, Mann Ward, 275 Bancroft Road, London, E1 4DG, UK

[3]Department of Respiratory Medicine, University Hospital of North Staffordshire, Stoke-on-Trent, Staffordshire, ST4 6QG, UK

*Author for correspondence:
Tel.: +44 0 1782 555261
Fax: +44 0 1782 747319
n.r.forsyth@keele.ac.uk

‡Authors contributed equally

Human mesenchymal stem cells (hMSC) are multipotent progenitor cells that can be isolated from multiple tissues including, among others, bone marrow and adipose tissues. MSC were first identified and described over half a century ago and their differentiation capacity and immunophenotype have since undergone rigorous examination [1,2]. hMSC have a generally accepted inherent mesodermal differentiation capacity and the potential for neuronal lineage generation. Immunophenotyping has produced a broad panel of positive and negative markers which characterize a cell as a mesenchymal stem cell, although practicalities (e.g., cell numbers, antibody availability) limit the number of markers which are routinely used for evaluation [3].

The wealth of experience surrounding hMSC from bone marrow (BMhMSC) culture and characterization has unsurprisingly led to the emergence of a broad range of clinical trial applications [4], including myocardial infarction, osteoarthritis, graft versus host disease, stroke, liver cirrhosis and both Type I and Type II diabetes [5]. Trial outcomes have been promising, although the mechanisms of action are unclear, and are accompanied by poor rates of post-transplant cell survival [6].

The terms physiological normoxia, reduced oxygen and hypoxia have been applied in studies to describe the culture atmosphere in investigations of the culture of cells in oxygen tensions lower than atmospheric levels, in other words, less than 21% O_2 [7–9]. The broad aim of these studies is to either recreate the niche-like conditions found in normal tissue compartments or those encountered in diseased tissue states such as tumor growth. The application of reduced oxygen tensions to stem cell research has produced descriptions of stem cell types including embryonic, hematopoietic, neuronal and hMSC [7–8,10–15]. Enhanced clonogenicity, reduced spontaneous differentiation, minimized epigenetic shift, transcriptional alterations, improved chromosomal stability

Future Medicine part of fsg

and increased numbers of differentiation target cells are consistently associated with culture of stem cells in reduced oxygen tensions (2–5% O_2) in comparison to an ambient air (20% O_2) setting [6,11,13,15–20].

Physiological normoxia within bone marrow is within the range of 2–8% O_2 where niches which support stem cell populations are hypothesized to occupy the lower end of the scale [21]. Routine isolation, culture, characterization and differentiation of hMSC have traditionally been performed under 20% O_2 conditions consistent with standard cell culture practice. More recently, several studies have described the application of physiological normoxia in these procedures. However, taken in cross-section, findings are inconsistent, frequently contradictory and no consensus outcome is reported [6,22–34].

Similarly, serum concentrations ranging from 5 to 20% serum have all been applied to hMSC isolation [6,11,22–24,26–30,35–38]. In addition to these, a number of studies have described the evaluation of serum-free approaches to the isolation of hMSC from bone marrow where control serum values ranging from 10 to 15% were employed [39–42]. In these studies it is not possible to ascertain the role, if any, of a reduced serum concentration versus serum-free counterparts in hMSC isolation. Identification of optimal serum concentrations for the isolation of BMhMSC would simplify and enable direct cross-comparison to take place in the absence of distinct variables.

An additional variable which has received some interest in evaluation of effects on hMSC biology and isolation is glucose. Cell culture media utilized in the isolation of hMSC from bone marrow is typically either 1 or 4.5 g/l glucose. Reports have indicated that hMSC display metabolic flexibility such that variations in glucose levels can alter pathway preference from oxidative phosphorylation to aerobic glycolysis, reversibly, depending on the prevailing environmental conditions [43–45]. Moreover it is also apparent that MSC require glucose to survive in ischemic environments but not in chronically low oxygen environments, reinforcing the previously described importance of metabolic flexibility in these cells [46].

Procedural differences in hMSC isolation through minimal alterations of oxygen level, base media, serum and substrate choice may all contribute to this variance in outcome but at levels which are currently unknown. We sought to clarify the impact of a subset of culture milieu factors; namely oxygen (0.2, 2 and 20% O_2); base media with variable glucose levels (1.0 and 4.5 g/l), due to its reported role in reduced oxygen environments; and serum (5 and 10% fetal bovine serum [FBS]), in the isolation of BMhMSC and identify mechanistic components, if appropriate. Here we have demonstrated that 2% O_2, 4.5 g/l glucose and 5% FBS was optimal versus other tested conditions in the isolation of BMhMSC. A 50% reduction of standard serum supplementation was also associated with a substantially enhanced frequency of BMhMSC isolation, whereas glucose levels played little role. We have further demonstrated that increasing the level of engineered oxygen control applied to the recovery of BMhMSC has a direct, positive, impact on the frequency of isolation. The reduction of re-oxygenation opportunities was associated with increased colony forming unit – fibroblastic (CFU-F) and BMhMSC cell number isolation in a 2% O_2 environment. Immunophenotypic and differentiation analysis confirmed that isolated cells were BMhMSC in all conditions tested. Transcriptional analysis identified differential expression in optimized versus nonoptimized isolation conditions where 83 transcripts displayed consistent differential expression associated with modulation of bone morphogenetic protein 2 (*BMP2*) and, putatively, chemokine (C-X-C motif) receptor 2 (*CXCR2*) signaling pathways. BMP2 supplementation was refractory to BMhMSC isolation in optimal conditions. Taken together these results indicate that an optimized culture media coupled to engineered oxygen control measures, such as workstations, enhance BMhMSC isolation putatively through downregulation of BMP2 signaling pathways.

Materials & method

BMhMSC isolation & culture methodology

BMhMSC were isolated from commercially sourced donor bone marrow aspirate retrieved from the iliac crest of 11 donors aged between 18 and 45 (Lonza, MD, USA). Bone marrow aspirates were transported under controlled temperature conditions (20°C) and supplemented with heparin to prevent coagulation. Aspirates were accompanied by mononuclear cell counts and were gently agitated to resuspend all cells prior to seeding. Seeding was performed within 48 h of aspiration where previous findings detail 80–90% mononucleated cell (MNC) viability [47]. BMhMSC isolation and expansion was performed using a modification of a previously described protocol which does not incorporate a separate MNC isolation step, for instance, density centrifugation [11,48]. Briefly, whole bone marrow was seeded at a density of 10^5 MNC/cm^2 on a minimum of six 10 ng/ml fibronectin-coated flasks per donor per condition; three flasks were Giemsa stained for CFU-F quantification and three flasks were used to determine final cell counts and for RNA isolation. Additional flasks were seeded for use in differentiation assays or immunofluorescence. MNC were seeded into either high-glucose Dulbecco's-Modified Eagles Media

(4.5 g/l, G_H) or low-glucose Dulbecco's-Modified Eagles Media (1.0 g/l, G_L). Media was supplemented with either 10% (S_H,) or 5% (S_L) batch-tested FBS (Lonza), 100 U/ml penicillin, 100 U/ml streptomycin, 1% NEAA and 1% L-Glutamine (all Lonza, UK). Penicillin was included initially to counteract the inherent infection risk associated with the culture of primary materials. Media was passively deoxygenated in hypoxic conditions post cell seeding to bypass inconsistencies associated with preincubation of media in hypoxic conditions. After 7 days half of the culture medium was replaced with fresh antibiotic-free media; after 14 days media was replaced completely again without the inclusion of antibiotic. At day 21, the flasks were washed and fixed with 10% Neutral Buffered Formalin for CFU-F Giemsa stain-based quantification, or harvested for cell counting and RNA isolation. Day 21 cells were classified as passage 0 (P0). Giemsa staining was performed as described elsewhere [47]. CFU-F numbers were determined microscopically where Giemsa stained clusters of greater than 16 cells were positively scored [49]. For immunofluorescence characterization fresh bone marrow aspirate was seeded into 24-well plates at the seeding density indicated above. BMhMSCs were recovered over 21 days and then washed and fixed, as P0, and as described below. For differentiation potential determination BMhMSC were again isolated as described above and then stimulated as described below. Cells were not passaged for initial characterization experiments and are therefore classified as P0. BMhMSC recovery was performed in either humidified incubators or hermetic workstations set at 37°C, 7% CO_2 and varying O_2 concentrations: either 20% O_2 (standard incubator), 2% O_2 (single-use incubator, multiuser incubator, workstation [RS Biotech, UK and Ruskinn, UK], flushbox [15]) and 0.2% O_2 (flushbox). Reductions in O_2 were achieved by either controlled N_2 purging of standard tri-gas incubators (RS Biotech, Irvine, UK) using a N_2 generator (Peak Scientific, UK) or by purging flushboxes with premixed gas. For exogenous BMP2 supplementation 50 ng/ml BMP-2 (Gibco/Life Technologies, Paisley, UK) was added over the duration of CFU-F recovery, as described above. Cell counts were performed with a hemocytometer where trypan blue exclusion was included as a measure of viability.

Immunofluorescence

Fresh bone marrow was seeded into 24-well plates, as described above, and P0 BMhMSCs recovered over a 21-day period. Cells were fixed with 4% paraformaldehyde, blocked with 3% BSA (bovine serum albumin) and incubated overnight at 4°C with anti-CD44, anti-CD90, anti-CD146, anti-CD14, anti-CD19 and anti-STRO-1 primary antibodies (Human MSC Characterization Kit. Cat. No. SCR067, Millipore, Watford, UK). Secondary antibody incubation was at room temperature for 2 h before washing and mounting in Dapi-Vectashield Secondary antibodies anti-mouse IgG-NL557 (CD44, CD90, CD146, CD144, CD19) and antimouse IgM-NL493 (STRO-1). Representative images are presented for all of the above CD markers. For quantitative analysis, STRO-1 positive BMhMSCs were counted per field at 20× magnification. Three fields were counted from each well of a triplicate experiment on P0 and passage 1 (P1) BMhMSCs. In this instance BMhMSC were passaged at Day 21 by first washing with PBS followed by exposure to Trypsin/EDTA until cells detached. After enzymatic quenching with excess medium BMhMSC were centrifuged at 1000 rpm for 2 min and then seeded at 1:2 passage ratios. Reseeded cells were classed as P1 and were fixed, described above, after a further 14 days in culture.

Differentiation

Differentiation assays were performed as described elsewhere [17]. In brief, BMhMSC were isolated as described above and after 21 days media was replaced with either osteogenic, adipogenic or chondrogenic medium and cells maintained for a further 21 days before being fixed and characterized by histological staining. To detect osteogenesis, fixed cells were immersed in 1% Alizarin Red solution for 5 min followed by washes in distilled water until effluent was clear. To detect chondrogenesis, fixed cells were stained overnight in 0.1% Alcian Blue solution at pH 1.5 followed by rinses in distilled water to remove excess stain. Adipogenesis was detected by first rinsing fixed cells in 60% isopropyl alcohol before staining with a 0.18% Oil Red O/60% isopropyl alcohol solution for 5 min. Samples were washed in tap water until clear. Differentiation was detected microscopically and images captured where positive staining had occurred. For chondrogenesis, a micromass approach was adopted according to the manufacturer's instructions (StemPro Chondrogenesis Differentiation Kit [Invitrogen, Paisley, UK]). Differentiated cell image capture was performed with either a 10× (osteogenesis) or 40× objective (chondrogenesis and adipogenesis) on an Eclipse TS100 (Nikon) with an EOS 400D (Canon). Immunofluorescence images were captured with a 20× objective on an Eclipse Ti-S (Nikon) inverted microscope system with a DS-Fi1 (Nikon) high-definition color camera head using appropriate filter sets.

Microarray analysis

RNA was extracted from day 21, P0, harvested BMhMSC cell pellets archived from a single donor recovered in $G_HS_LO_{2\%WS}$ (×4 individual cell pellets),

$G_HS_LO_{2\%}$ (×4 individual cell pellets) and $G_HS_HO_{21\%}$ (×4 individual cell pellets) with lysis, homogenization and extraction performed according to manufacturer's guidelines (QIAShredder; RNeasy [Qiagen, Manchester, UK]). While clear advantages exist to performing microarray-based analysis across multiple donors, the selection of a single donor approach for candidate identification followed by multiple donors for verification purposes allowed similar stringency levels to be applied. RNA was first rRNA reduced followed by first and second strand cDNA synthesis and transcription. cRNA was subsequently cleaned, transcribed into cDNA, cleaned again, fragmented, labeled and hybridized overnight onto Exon 1.0ST arrays before washing, staining and scanning. All procedures were performed according to manufacturer's guidelines (Affymetrix). Hybridization, washing and scanning were performed using a GeneChip Hybridisation Oven 640, GeneChip Fluidics Station 450, and 7G GeneChip Scanner 3000 (Affymetrix).

Quantitative RT-PCR was performed against a discrete selection of genes which displayed differential expression in response to varying O_2 conditions to provide secondary, independent, verification of array data. qRT-PCR was performed using triplicate independent RNA isolates from hMSC isolated from three independent donors. Primer sequences and candidate gene were; *CXCL1* (5′-TCACCCCAAGAACATCC-3′ [Forward], 5′- GCTGAACAGTGACAAATCC -3′ [Reverse]); *PTGIS* (5′-TGTCTTCCACACCTTTCG -3′ [Forward], 5′- GGAGATGGGTGTGTCAGA-3′ [Reverse]); and *ACAN* (5′- TCTGAGGGTCATCACTGC-3′ [Forward], 5′- CTTCTCCTTGGACACACG-3′ [Reverse]). Expression was normalized against *GAPDH* (5′- GACTTCAACAGCGACACC-3′ [Forward], 5′- GCAAGAGCACAAGAGGAA-3′ [Reverse]). Genes were randomly selected for inclusion in this analysis from the 80-gene BMhMSC core transcriptional footprint.

Bioinformatics analysis

Cell intensity (CEL) files were subsequently exported from the Affymetrix Workstation and imported into DChip (2008 build) for quality control and normalized using median probe intensity as baseline array. Exon level expression data were first generated and then exported as U133 Plus 2.0 data using the supplied mapping file. Data sample groupings were then established, values sorted according to expression and selected based on fold changes of >2 and statistical significance, $p < 0.05$. Ontological and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed on the DAVID Bioinformatics Resource using appropriately selected identifiers and background formats [50,51].

Statistical analysis

Statistical analysis was performed using Minitab 15.0 (Coventry, UK), Graphpad Prism 4 (La Jolla, CA, USA) and Origin 8.1 (Northampton, MA, USA) with further analysis performed in Microsoft Excel Spreadsheets application. A Paired Student's *t*-test was used in comparative evaluation of CFU-F and BMhMSC cell yields with $p < 0.05$ considered significant. Microarray data was analyzed using dChip software with the DAVID bioinformatics resource and a Paired Student's *t*-test used to identify statistically significant variations in gene expression.

Results

Reports describing the efficacy of isolation of hMSC from bone marrow aspirates vary in the application of reduced oxygen, serum concentration and glucose level. To establish a background methodology for the recovery of BMhMSC we tested a matrix of glucose (G); High (G_H), 4.5 g/l and Low (G_L), 1 g/l, serum (S); High (S_H) 10% and Low (S_L), 5% and oxygen (O); 20% O_2 ($O_{20\%}$), 2% O_2 ($O_{2\%}$), and 0.2% O_2 ($O_{0.2\%}$). Determination of the optimal matrix point, as defined by the greatest number of CFU-F, was made by seeding bone marrow aspirate into each grouping in triplicate from each of three independent donors. CO_2/N_2 incubators remained unopened except when media changes were required during the CFU-F recovery process unless otherwise indicated.

Quantification of CFU-F showed that cultivation and expansion under standard conditions ($G_HS_HO_{20\%}$) yielded a mean of 28.6 CFU-F/75 cm^2, while optimal CFU-F recovery was observed in $G_LS_LO_{2\%}$ and $G_HS_LO_{2\%}$ (48.3 CFU-F/75 cm^2 and 49.3 CFU-F/75 cm^2 respectively) (Figure 1A). Mean values were calculated from triplicate samples for each donor; mean values from each donor were then pooled to generate an overall mean as presented in Figure 1. Widespread variance was observed between the three initial donors ($p < 0.0001$) resulting in noticeable differences in CFU-F recovery which did not achieve significance levels. The proliferative potential of BMhMSC recovered at differing matrix points was broadly reflective of initial CFU-F yield with minor exceptions (e.g., $G_LS_LO_{20\%}$, $G_LS_LO_{2\%}$ and $G_LS_LO_{0.2\%}$ underwent rapid replicative exhaustion [data not shown]). We next tested bone marrow aspirate from a further six independent donors seeded into $G_HS_HO_{20\%}$, $G_HS_LO_{20\%}$ and $G_HS_LO_{2\%}$ representing standard, intermediary (where serum was the solitary component altered), and optimal recovery conditions, respectively, in triplicate. Increasing sample numbers again produced an increase in variability, with $G_HS_HO_{20\%}$ on this occasion yielding a greater number of CFU-F than $G_HS_LO_{20\%}$ (55.25 CFU-F/75 cm2 vs 33 CFU-F/75 cm^2)

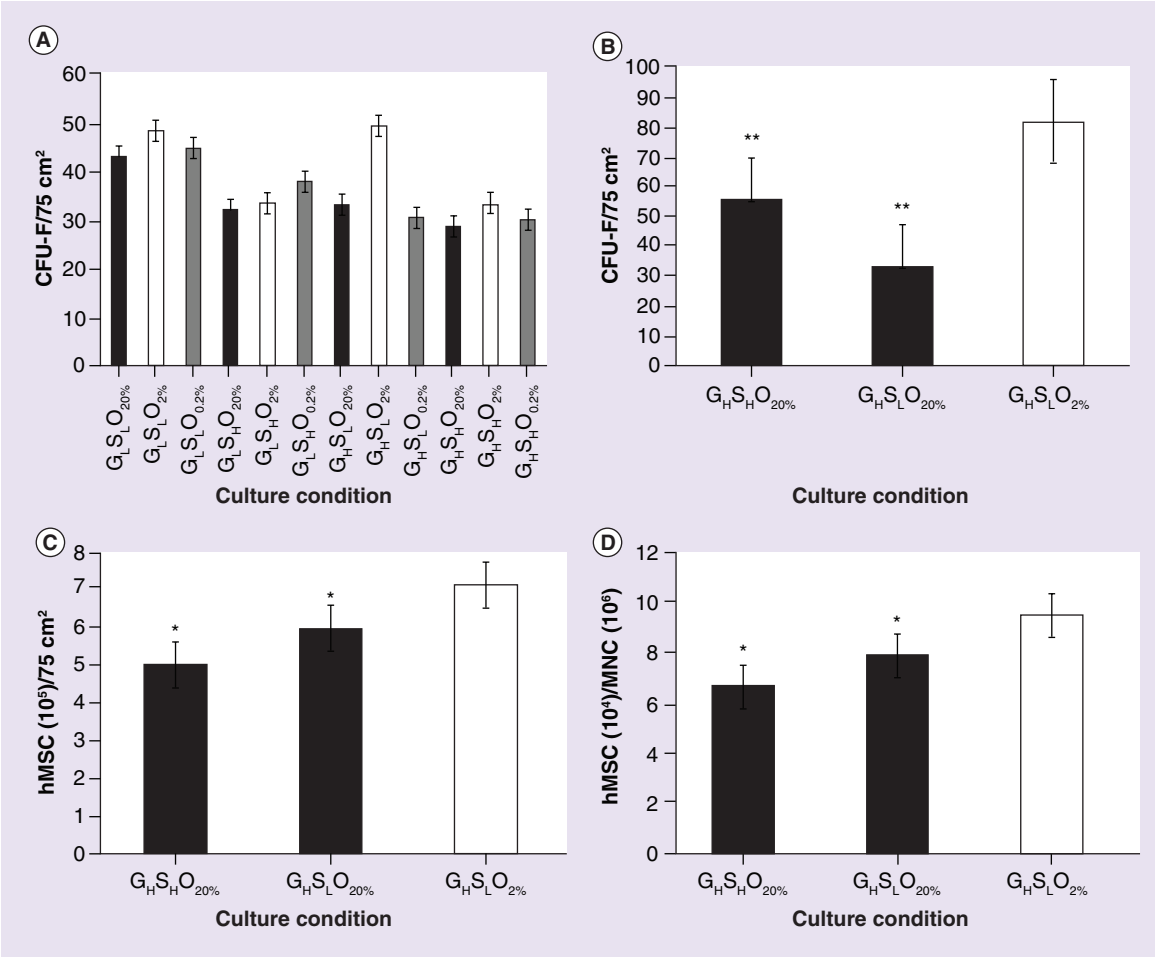


Figure 1. Physiological normoxia and serum synergistically promote human mesenchymal stem cells from bone marrow isolation. (A) Bone marrow aspirate was seeded across a matrix of conditions including high (4.5 g/l) and low (1.0 g/l) glucose media (G_H and G_L), high (10%) and low (5%) serum (S_H and S_L), and physiological hyperoxia (20% O_2), normoxia (2% O_2) and hypoxic (0.2% O_2) culture atmosphere oxygen concentrations ($O_{20\%}$, $O_{2\%}$, $O_{0.2\%}$). Optimal human mesenchymal stem cells from bone marrow recovery was associated with G_LS_L and $S_LO_{2\%}$ combinations where $G_HS_LO_{2\%}$ and $G_LS_LO_{2\%}$ supported maximum human mesenchymal stem cells from bone marrow CFU-F yields (n = 3 independent donors in triplicate). G_LS_L did not promote subsequent human mesenchymal stem cells from bone marrow expansion. **(B)**, **(C)** and **(D)** describes increased CFU-F per T75 flask, human mesenchymal stem cells from bone marrow per T75 flask and human mesenchymal stem cells from bone marrow (10^4) per MNC (10^6) seeded, respectively, for $G_HS_LO_{2\%}$ versus $G_HS_LO_{21\%}$ and $G_HS_HO_{21\%}$ (n = 6 independent donors in triplicate). Error bars indicate standard error. *$p < 0.05$ versus $G_HS_LO_{2\%}$, **$p = 0.001$ versus $G_HS_LO_{2\%}$.
CFU-F: Colony Forming Unit - Fibroblastic; hMSC: Human mesenchymal stem cells; MNC: Mononuclear cell.

though $G_HS_LO_{2\%}$ remained optimal (80.9 CFU-F/75 cm^2, $p = 0.001$ vs $G_HS_HO_{20\%}/G_HS_LO_{20\%}$) (Figure 1B). However, irrespective of CFU-F frequency, BMhMSC recovery per flask, expressed as either per T75 flask or per 10^6 MNC seeded, followed a clear hierarchy of $G_HS_LO_{2\%} > G_HS_LO_{20\%} > G_HS_HO_{20\%}$ (Figure 1C & D, $p < 0.05$ vs $G_HS_HO_{20\%}/G_HS_LO_{20\%}$).

We next sought to establish whether variations in the degree of hermetic (airtight) control had any direct consequence on BMhMSC yield. We seeded MNC from a single donor into flasks contained in either: a single-use, unopened, CO_2/N_2 incubator; a multiuser, CO_2/N_2 incubator; a CO_2/N_2 workstation; a multiuser CO_2/N_2 incubator with media changes in a CO_2/N_2 workstation; a predetermined CO_2/N_2 gas concentration flushbox system [16]; and a multiuser CO_2 incubator (Figure 2A). A single donor was selected at this point due to the statistical strength associated with the use of $G_HS_LO_{2\%}$ (Figure 1B–D). The frequency and nature of ambient oxygen exposure events are detailed in Table 1. Statistical evaluation was performed with a Paired Student's *t*-test versus 20% O_2 in all instances. All media changes were performed in a standard Class II Biological Safety Cabinet unless otherwise stated. Surprisingly, the multiuser, CO_2/N_2 incubator (125.3 CFU-F/T75 cm^2) and the multiuser

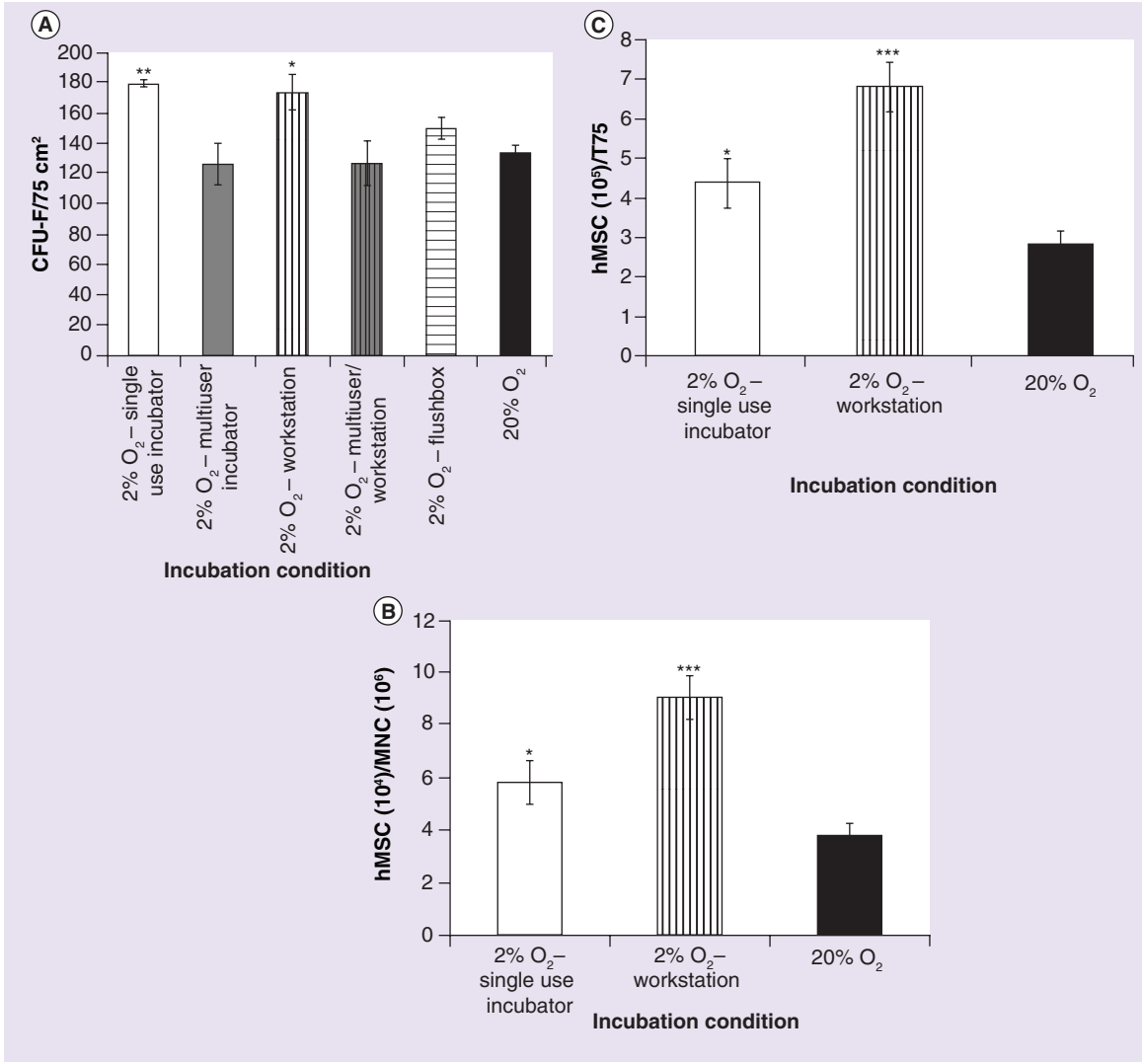


Figure 2. Repeated air oxygen exposure is refractory to human mesenchymal stem cell recovery. Bone marrow aspirate was seeded into flasks containing $G_HS_LO_{2\%}$ across a number of systems in common use for control of cell culture O_2 levels. **(A)** Human mesenchymal stem cells from bone marrow were recovered in either standard incubator (20% O_2) (black bar), flushbox (2% O_2) (horizontal striped bar), single user tri-gas incubator (2% O_2) (open bar), multiuser tri-gas incubator (2% O_2) (gray bar), workstation (2% O_2) (vertical striped bar) and multiuser tri-gas incubator with workstation for handling (2% O_2) (vertical striped gray bar). CO_2 was maintained at 7% in all instances with N_2 to balance and temperature constant at 37°C. Unless otherwise stated all culture handling was performed rapidly in a standard biological safety cabinet. **(B&C)** describe human mesenchymal stem cells from bone marrow recovered per mononucleated cell seeded and human mesenchymal stem cells from bone marrow recovered per T75 flask where enhanced isolation was observed in 2% O_2 workstation versus 2% O_2 single user tri-gas incubator versus 20% O_2 standard incubator (n = 3).
Error bars indicate standard of error. *p = 0.01, **p = 0.001, ***p = 0.0001.
CFU-F: Colony forming unit - fibroblastic; hMSC: Human mesenchymal stem cell.

CO_2/N_2 incubator with media changes in a CO_2/N_2 workstation (126 CFU-F/T75 cm^2) were comparable to the multiuser CO_2 incubator (133 CFU-F/T75 cm^2) in terms of BMhMSC CFU-F yield (p = 0.53 and p = 0.47, respectively, vs 20% O_2). As indicated in Table 1 this suggests that frequent ambient oxygen exposure events caused by incubator door opening during the recovery period can result in reductions in CFU-F yield. The optimal environmental control apparatus when used in combination with G_HS_L was either the single-user, unopened, CO_2/N_2 incubator (178.3 CFU-F/T75 cm^2, p = 0.001 vs 20% O_2) or the CO_2/N_2 workstation (172.6 CFU-F/T75 cm^2, p = 0.01 vs 20% O_2). The flushbox system yield was comparable to the above (149 CFU-F/T75 cm^2, p = 0.063) but due to the inherent space constraints on the system and the susceptibility to mold contamination the system was not included in further analysis. The

Table 1. Cell culture systems and air exposure events.

	CO_2 incubator	Multiuser, CO_2/N_2 incubator	CO_2/N_2 flushbox system	Single-use, CO_2/N_2 incubator	CO_2/N_2 workstation	Multiuser CO_2/N_2 incubator and CO_2/N_2 workstation
CO_2 (%)	7%	7%	7%	7%	7%	7%
O_2 (%)	20%	2%	2%/0.2%	2%	2%	2%
Air exposure events (per week)	All	11	2	1	0	11
Nature and duration of air exposure events	Incubation (constant), media changes (5 min)	Incubator door openings (~10 s), media changes (5 min)	Media changes (5 min)	Media changes (5 min)	None	Incubator door openings (~10 s)
Total time of air exposure over recovery period (~)	3 weeks	10–15 min	10 min	10 min	None	5–10 min

Human mesenchymal stem cells were cultured in a range of incubators with and without oxygen concentration control. Air exposure events relate to incubator openings (CO_2 and multiuser) and media changes in biological safety cabinets (CO_2, multiuser, flushbox, single use).

yields of BMhMSC CFU-F were comparable between the two optimal systems tested. The CO_2/N_2 workstation (2% O_2WS) yielded a substantially greater number of BMhMSC per 10^6 MNC than the single-user, unopened, CO_2/N_2 incubator (2% O_2) (9×10^4 vs 5.83×10^4, respectively) ($p = 0.0001$ and $p = 0.01$, respectively, vs 20% O_2) (Figure 2B). This translates to a cell yield in a CO2/N2 workstation of 6.8×10^5 per T75 flask (Figure 2C). In all subsequent experiments, BMhMSC isolated in single-user, unopened, CO_2/N_2 incubator (2% O_2) are referred to as $G_HS_LO_{2\%}$ whereas BMhMSC isolated in the CO_2/N_2 workstation (2% O_2WS) are referred to as $G_HS_LO_{2\%WS}$. In real terms the primary difference between the two optimal systems ($G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$) was approximately 10 min of air exposure during media changes, which were performed in a biological safety cabinet, to $G_HS_LO_{2\%}$ emphasizing the importance of continuous oxygen control during the recovery of BMhMSC.

Functional characterization of hMSC requires evidence of immunophenotype and differentiation capacity [2]. As anticipated adipogenic, osteogenic and chondrogenic differentiation capacity were evident in BMhMSC irrespective of isolation environment (Figure 3A). The efficiency of differentiation appeared altered but was not quantified; adipogenesis and osteogenesis levels were reduced whereas chondrogenesis was enhanced in $G_HS_LO_{2\%}$ versus $G_HS_HO_{20\%}$. Adipogenic and osteogenic differentiation levels of $G_HS_LO_{2\%}$ recovered BMhMSC were restored by switching into 20% O_2 for the duration of the differentiation time course. Immunophenotypic characterization demonstrated that, irrespective of the isolation environment, BMhMSC displayed strong expression of STRO-1, CD44, CD90 and CD146 and absent or limited expression of CD14 and CD19 (Figure 3B). STRO-1 expression was significantly higher in $G_HS_LO_{2\%}$ than in $G_HS_HO_{20\%}$ at P0 (95% $G_HS_LO_{2\%}$ vs 79% $G_HS_HO_{20\%}$, $p < 0.001$) but expression levels rapidly dropped in all conditions with subsequent passage and culture (P1) (Figure 3C & D). These characterizations confirm that cells isolated under $G_HS_LO_{2\%}$ are BMhMSC in both 2% O_2 and 2% O_2WS.

Global transcriptome analysis provides a powerful interrogational tool for the rapid identification of transcript alterations and representative pathway bias in biological samples [8,19,52–54]. The frequencies of BMhMSC CFU-F isolation were similar in 2% O_2 and 2% O_2WS which led us to hypothesize that a core set of transcripts would be differentially expressed in comparison to $G_HS_HO_{20\%}$. To test this hypothesis we isolated BMhMSC from a single bone marrow donor in either $G_HS_LO_{2\%WS}$, $G_HS_LO_{2\%}$ or $G_HS_HO_{20\%}$ conditions, in quadruplicate flasks. A single donor was selected for candidate gene identification with additional donors included for the later candidate verification (qRT-PCR) and mechanistic determination. RNA was isolated from each independent replicate flask and maintained as a unique experimental replicate throughout processing steps and hybridized to individual Exon 1.0ST array chips. We identified 71 and 68 downregulated probe sets in $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$, respectively versus $G_HS_HO_{20\%}$ which correlated to 61 unique genes in each while 16 were held in common (Figure 4A, Supplementary Tables 1 & 2, Supplementary Dataset 1; see online at: www.futuremedicine.com/doi/full/10.2217/RME.14.67). We next identified 226 and 232 upregulated probesets in $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$,

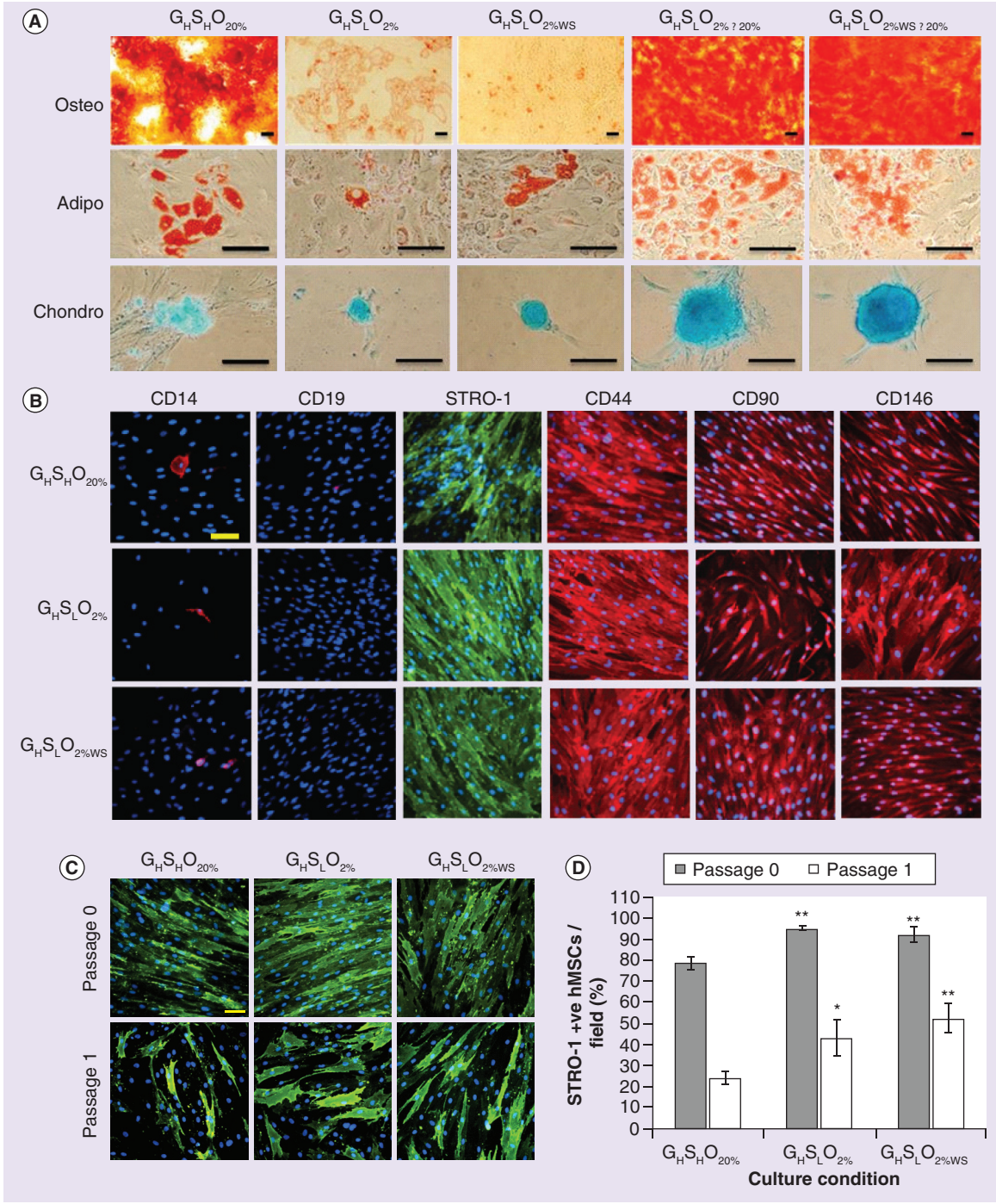


Figure 3. Multipotentiality and immunophenotypic retention of human mesenchymal stem cell isolated in optimized conditions. (A) Osteo, chondro and adipo differentiation in $G_HS_HO_{21\%}$ isolated hMSC versus $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ isolated hMSCs from bone marrow display broad multipotential differentiation retention with anticipated inhibition and enhancement as reported previously. Reductions in adipo and osteo differentiation are recovered by switching hMSCs from bone marrow back into 20% for differentiation ($G_HS_LO_{2\%\rightarrow20\%}$ and $G_HS_LO_{2\%WS\rightarrow20\%}$). **(B)** Immunophenotypes of hMSCs from bone marrow isolated in either $G_HS_HO_{20\%}$, $G_HS_LO_{2\%}$ or $G_HS_LO_{2\%WS}$ are broadly similar. Individual CD markers tested are indicated on the left-hand side of panels. **(C&D)** Sub-population of STRO-1 expressing hMSCs from bone marrows in passage 0 and passage 1 cells detected by **(C)** fluorescent immunocytochemistry and **(D)** quantified (n = 3). Nuclei are counterstained with 4′,6-diamidino-2-phenylindole (DAPI) . Scale bar indicates 100 μm throughout.
*$p < 0.05$ versus $G_HS_HO_{20\%}$; **$p < 0.01$ versus $G_HS_HO_{20\%}$ (Passage 0 or Passage 1).
Adipo: Adipogenic; Chondro: Chondrogenic; hMSC: Human mesenchymal stem cell; Osteo: Osteogenic.

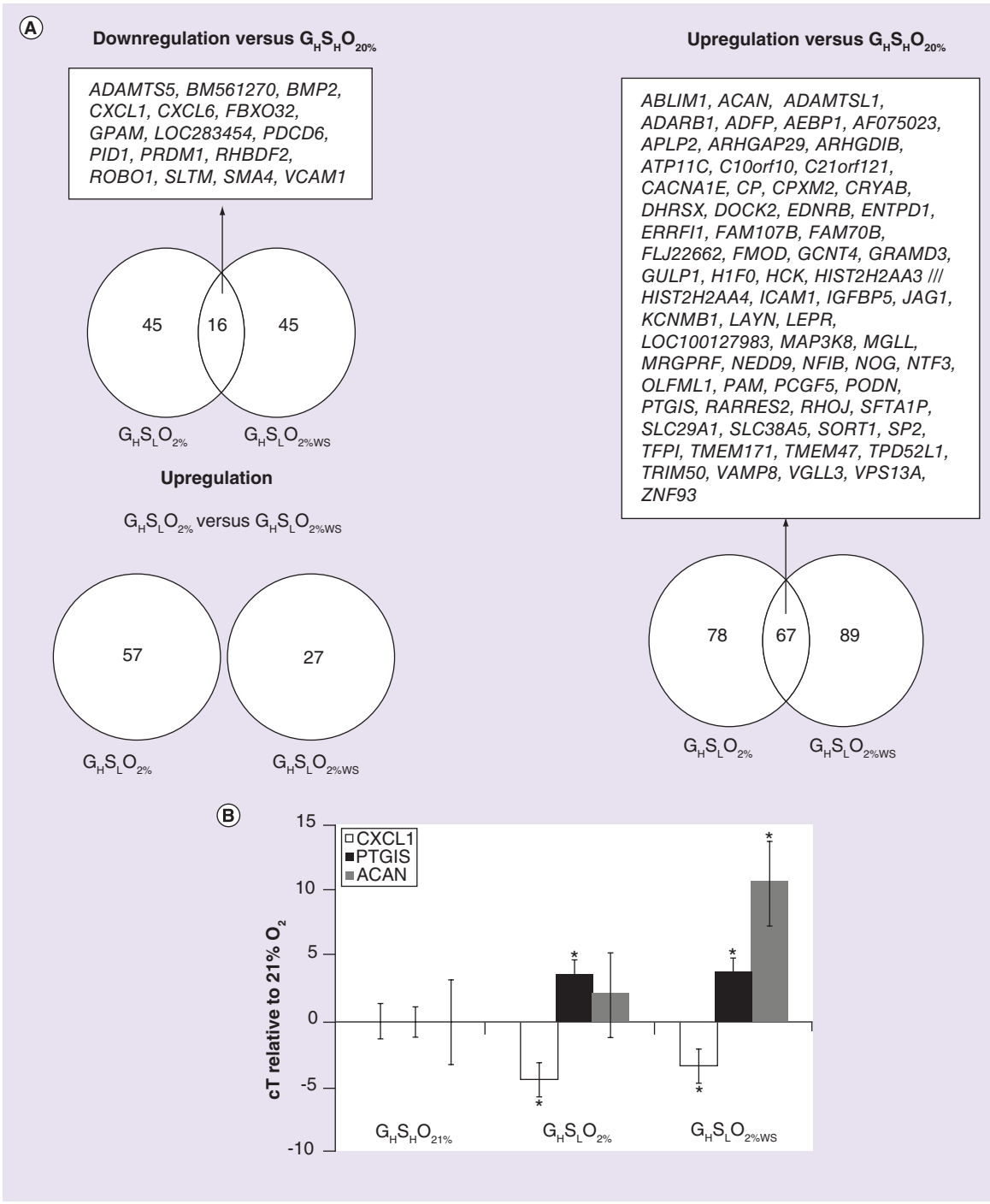


Figure 4. Transcriptome analysis identifies differentially expressed genes in $G_HS_HO_{20\%}$ versus $G_HS_LO_{2\%}$ isolated human mesenchymal stem cells from bone marrow. Human mesenchymal stem cells from bone marrow were lysed after 3 weeks recovery, RNA extracted and analysed using Exon1.0ST arrays before being exported as U133 Plus2.0 data. **(A)** $G_HS_LO_{2\%}$ isolated human mesenchymal stem cells from bone marrow displayed downregulation of 61 genes in comparison to $G_HS_HO_{21\%}$ where 16 were held in common between 2% O_2 workstation and single-use 2% O_2 incubator. Upregulation of 145 and 156 genes were noted in $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ isolated human mesenchymal stem cells from bone marrow versus $G_HS_HO_{20\%}$ isolates where 67 were held in common. $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ isolated human mesenchymal stem cells isolated human mesenchymal stem cells from bone marrow displayed distinct differential gene expression when directly compared with no apparent overlap. **(B)** QRT-PCR provided confirmation of differential expression of *CXCL1*, *PTGIS* and *ACAN*.
Error bars indicate standard error; *p ≤ 0.005 versus $G_HS_HO_{21\%}$.
See also Supplementary Tables 1–4 and Supplementary Dataset 1.

respectively versus $G_HS_HO_{20\%}$ which correlated to 145 ($G_HS_LO_{2\%}$) and 156 ($G_HS_LO_{2\%WS}$) unique genes in each where 67 were held in common (Figure 4A, Supplementary Tables 3 & 4, Supplementary Dataset 1). This identified a core set of 16 downregulated and 67 upregulated transcripts in $G_HS_LO_{2\%}$ versus $G_HS_HO_{20\%}$ hMSCs (Figure 4A). Subsequent analysis identified 63 and 37 probe sets differentially overexpressed between $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$, respectively which correlated to 57 ($G_HS_LO_{2\%}$) and 27 ($G_HS_LO_{2\%WS}$) unique genes with no observed overlap (Figure 4A). qRT-PCR provided confirmation of transcriptional changes for a subset of differentially expressed genes from the core set (*CXCL1*, *PTGIS* and *ACAN*) (Figure 4B). qRTPCR was performed using triplicate independent RNA isolates from BMhMSC isolated from three independent donors.

We next sought to identify whether the differential expression of transcripts between isolation environments would translate into over- or under-representation across the GO hierarchies. No correlation was observed in underexpressed transcripts with cellular component (CC) GO terms in either $G_HS_LO_{2\%}$ or $G_HS_LO_{2\%WS}$; however, a significant under-association was noted with cytokine activity (molecular function) and with six Biological Process (BP) (Figure 5A, Supplementary Tables 5 & 6). Over-expressed transcripts in $G_HS_LO_{2\%}$ or $G_HS_LO_{2\%WS}$ were over-represented in six CC terms (all related to plasma membrane or extracellular part), four BP (all related to either development or adhesion), and one molecular function (small GTPase regulator activity) (Figure 5B, Supplementary Tables 7 & 8). No overlapping over-representations were observed in the analysis of differentially expressed transcripts between $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ (Figure 5C). Final corroboration of the validity of the core group of differentially expressed transcripts was provided by KEGG pathways analysis (Figure 6). Strong and significant association was noted between differentially downregulated transcripts in $G_HS_LO_{2\%}$ versus $G_HS_HO_{20\%}$ and the cytokine–cytokine receptor interaction (CCRI) in $G_HS_LO_{2\%}$ ($p = 0.067$), $G_HS_LO_{2\%WS}$ ($p = 0.04$), $G_HS_LO_{2\%}/G_HS_LO_{2\%WS}$ core downregulated set ($p = 0.035$). Although an increase in CCRI associations was noted in $G_HS_LO_{2\%WS}$ (*CXCL1*, *CXCL6*, *ACVR2*, *BMPR1A*, *BMP2*) compared with $G_HS_LO_{2\%}$ (*CXCL1*, *CXCL6*, *IL8*, *BMP2*) or in $G_HS_LO_{2\%}/G_HS_LO_{2\%WS}$ downregulated core set (*CXCL1*, *CXCL6*, *IL8*, *BMP2*), the associations were held in common with signaling through either *IL8* (*CXCR2*) receptors or through *BMP2* (Figure 6A). The differential over-expression of *NOG* in the $G_HS_LO_{2\%}/G_HS_LO_{2\%WS}$ upregulated core set reinforces the likely importance of repression of *BMP2* signaling in the enhanced isolation and proliferative expansion of BMhMSC in $G_HS_LO_{2\%}$ (Figure 6B). To evaluate the role of *BMP2* transcriptional repression in BMhMSC isolation, we continuously supplemented $G_HS_LO_{2\%}$ with BMP2 in both $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ during recovery of BMhMSC over 21 days from an additional bone marrow aspirate. Exogenous supply of BMP2 protein induced a 40 and 35% reduction in $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ recovered BMhMSC respectively ($p \leq 0.007$) (Figure 7).

Discussion

Several often contradictory reports describe the isolation and/or subsequent culture and characterization of hMSC in reduced oxygen tensions. Quantification of CFU-F suggests that in reduced oxygen tensions colony numbers and colony size can be either reduced, increased, or stay the same when compared with ambient air recovered hMSC [22–23,26,30,33–34]. Less contradictory, though still not consistent, are the descriptions of increased cell numbers and decreased expansion times in reduced oxygen versus ambient air recovered cells [6,11,24,27–29,35–38]. Through a combination process of optimized culture conditions coupled to examination of oxygen control equipment, we have determined that much of the previous variation in hMSC CFU-F frequency can be attributed to the equipped level of oxygen control: tighter control via removal of multiple door openings during the recovery period appeared to associate with a significantly increased frequency of CFU-F isolation and absolute BMhMSC numbers.

There is a major interpretative difficulty which emerges as the broad range of isolation parameters which are described for hMSC becomes apparent. For instance, oxygen tensions and serum concentrations ranging from 1 to 5% O_2 and 5 to 20% serum, respectively, have all been applied to previous descriptions of reduced oxygen hMSC isolation [6,11,22–24,26–30,35–38]. In this study mean colony yield was not significantly different between isolation conditions which incorporated either G_H or G_L (mean CFU-F's of 34 and 40, respectively), S_H or S_L (mean CFU-F's of 33 and 41, respectively), or either of $O_{20\%}$, $O_{2\%}$ or$O_{0.2\%}$ (mean CFU-F's of 34, 41 and 36, respectively) (Figure 1A). Differences were apparent but a combination of factors ($G_HS_LO_{2\%}$) was required to optimize CFU-F isolation to significant levels above other conditions (Figure 1B–D). The optimization step allowed the impact of engineered oxygen control measures to be explored with confidence. The subsequent incorporation of tighter oxygen control measures resulted in significant improvements in CFU-F isolation above those seen for medium/environment optimization alone (Figure 2). It is important to note that a strong

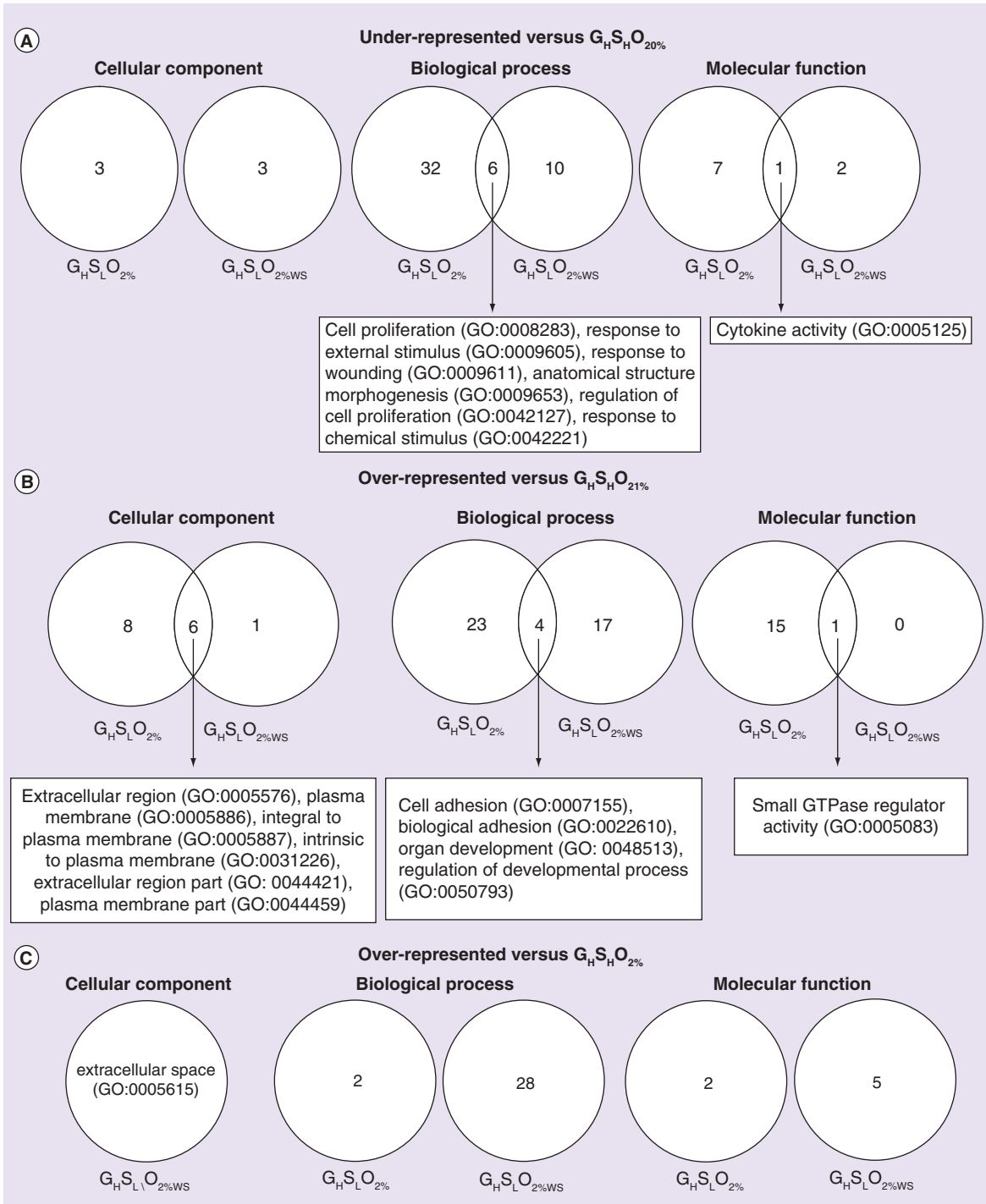


Figure 5. Gene ontology analysis identifies specific terms associated with differentially expressed genes in $G_HS_HO_{20\%}$ versus $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$. (A) Gene ontology (GO) terms significantly under-represented in $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ versus $G_HS_HO_{20\%}$ are identified in the extracellular region (CC), biological process (BP), and molecular function (MF) hierarchies. Common under-representation includes cytokine activity (molecular function), regulation of proliferation (BP), external stimuli response (BP) and shape development (BP) terms. **(B)** Over-representation versus $G_HS_HO_{20\%}$ was also noted across the three main hierarchies. Common terms included small GTPase regulation (molecular function), adhesion and development (both BP), plasma membrane and extracellular region (CC). **(C)** No common GO terms were noted for $G_HS_LO_{2\%}$ versus $G_HS_LO_{2\%WS}$. Values indicate number of GO terms associated per hierarchy per grouping. See also Supplementary Tables 5–8 for listings of all GO terms identified and Affymetrix probes and associated genes of all common identified GO terms.

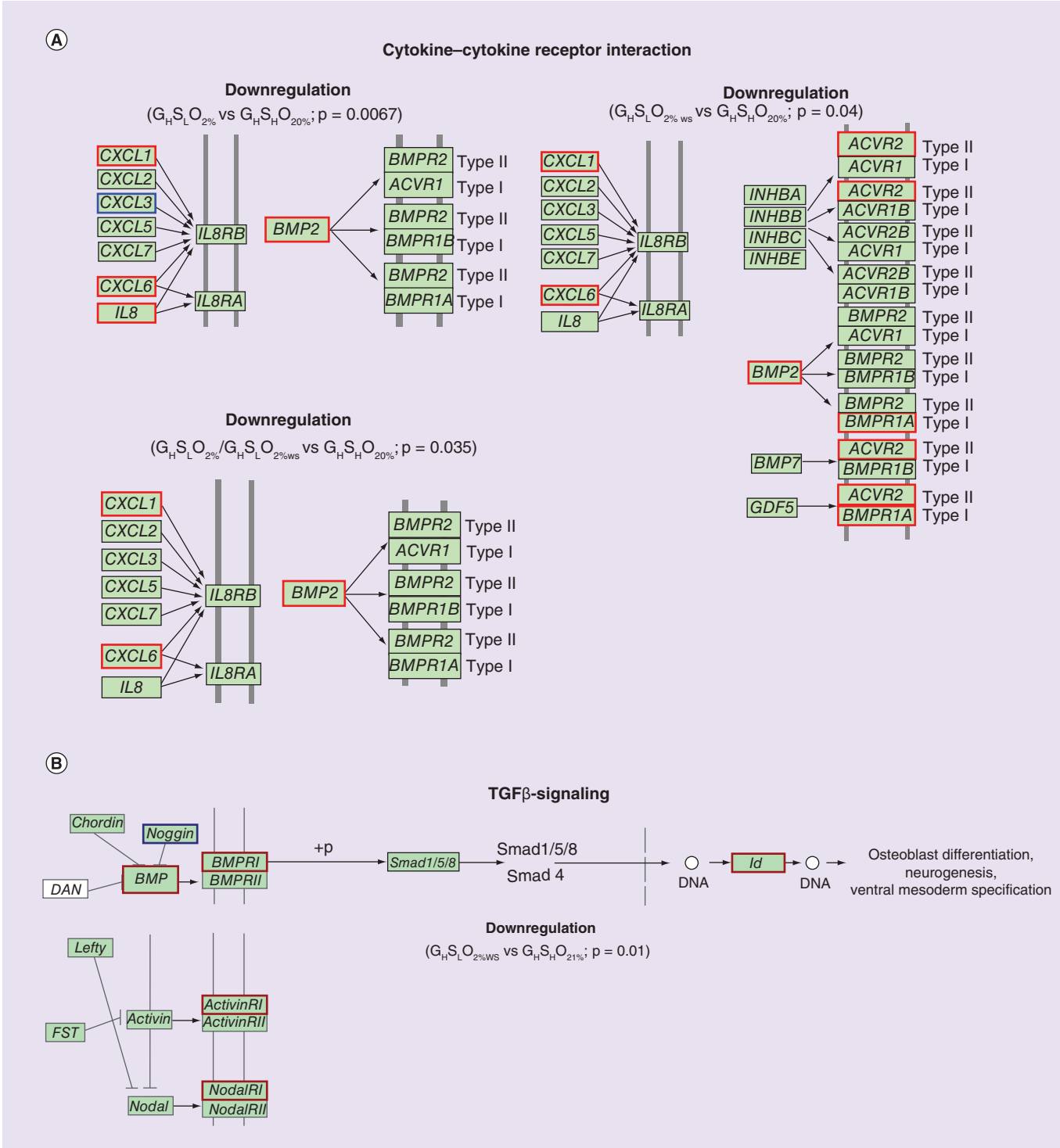


Figure 6. Differentially expressed genes associate with specific kyoto encyclopedia of genes and genome pathways. (A) Database for annotation, visualization and integrated discovery-based evaluation of differentially expressed genes revealed significant association of $G_HS_LO_{2\%}$ (p = 0.0067) and $G_HS_LO_{2\%WS}$ (p = 0.04) downregulated genes with the cytokine–cytokine receptor interaction pathway. This was highlighted by downregulation of both *BMP2* and *CXCR2*. Similarly DAVID-based evaluation of the core differential expression transcripts revealed a significant association with cytokine–cytokine receptor interaction pathway (p = 0.035) again through downregulation of *BMP2* and *CXCR2*. **(B)** DAVID analysis identified a significant association (p = 0.01) of $G_HS_LO_{2\%WS}$ versus $G_HS_HO_{20\%}$ differential expression with TGFβ through bone morphogenetic protein 2 (BMP) downregulation and noggin upregulation. Red boxed genes indicate downregulation in $G_HS_LO_{2\%}$ and/or $G_HS_LO_{2\%WS}$ as specified, blue boxes indicate upregulation.
For colour images please see online at: www.futuremedicine.com/doi/full/10.2217/RME.14.67

correlation emerged between a multiuser incubation environment and reduced BMhMSC yield. This was hypothesized to be in some part due to a cumulative effect of a repeated cycle of chamber reoxygenation, media reoxygenation, chamber deoxygenation and media deoxygenation and not due to air exposure during media changes (Figure 2 & Table 1).

Our experiments have adopted the D'Ippolito *et al.* method, with minor modifications in a slight reduction of maintenance O_2 used, and the adoption of a hermetically tight workstation for impact evaluation [11]. However, switching BMhMSC into a 2% FBS supplemented media for expansion did not yield immortal hMSC as previously reported [11]. As a direct consequence of multiparametric analysis, we concluded that $G_HS_LO_{2\%}$ is the optimum condition for BMhMSC recovery where serum and oxygen levels are the critical components.

Consensus immunophenotypic profiles of hMSC in reduced oxygen versus ambient air oxygen levels suggest a largely unchanged profile (CD13, CD29, CD73, CD90, CD105, CD166, CD271, and HLA-1) with some accompanying increases (CD106 and STRO-1) in surface antigen expression levels [23,35,55]. Nonquantitative (CD44, CD90, CD146) and quantitative (STRO-1) analysis of immunofluorescence images in our study is in broad agreement with these previous reports. A defining characteristic of hMSCs is their multipotent differentiation potential [2]. The effect of reduced oxygen culture on differentiation is described across multiple reports where general differentiation potential is retained but capacity frequently altered. Similarly, the above reports do not allow a reliable consensus on the effects of oxygen tension on differentiation potential. Most describe osteogenic potential of hMSC as reduced [6,24,27–29,31,33], while in others this osteogenic capacity is enhanced [26,32] or unchanged [54,56]. Adipogenic differentiation is similarly described as being enhanced [6,25–26,33] or reduced [24,27–28], while chondrogenic potential is generally referred to as enhanced [6,55,57–58]. We observed retention of osteogenic, adipogenic and chondrogenic differentiation potential in all conditions but some evidence of reduced osteogenic and adipogenic potential in hypoxic conditions. Further studies will be required to elaborate the role of oxygen and optimized culture conditions during differentiation.

Transcriptome determination provides an invaluable tool in developing mechanistic understanding in cell behavior and response to environment alteration. We identified a consensus optimized BMhMSC fingerprint of 80 differentially expressed (16 downregulated and 64 upregulated) genes. These transcripts were modulated in both 2% O_2 and 2% O_2WS, and over-represented in

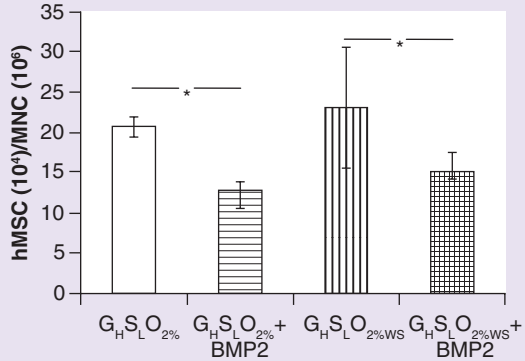


Figure 7. BMP2 inhibits human mesenchymal stem cells from bone marrow recovery in $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$.
hMSC from bone marrow recovery was evaluated in both $G_HS_LO_{2\%}$ and $G_HS_LO_{2\%WS}$ in the presence of 50 ng/ml BMP2.
*$p \leq 0.004$.
BMP2: Bone morphogenetic protein 2; hMSC: Human mesenchymal stem cell; MNC: Mononuclear cell.

identical ontology groups. A surprising paucity of data exists with which we can compare, contrast and evaluate our findings. Downregulation of transcripts associated with DNA metabolism, cell cycle progression and chromosome adhesion (*RRM2*, *XRCC2*, *KIF24*, *POLQ*, *E2F8*, *FANCD2*, *ESCO2*, *AVRKB*, *CENP2*, *MKI67*) and upregulation of transcripts associated with adhesion and metabolism pathways (*CD93*, *ESAM*, *VWF*, *PLVAP*, *ANGPT2*, *LEP*, and *TCF1*, *ZP1*, *GIMAP4*, *PCDH17*) was reported in P0 hMSC [23]. No conservation with consensus fingerprint was immediately apparent although 2% O_2 ($G_HS_LO_{2\%}$) BMhMSC display differential upregulation of *LEP*, albeit outside of the consensus fingerprint, whereas *LEPR* is within the consensus fingerprint. *LEPR* is homologous to members of the gp130 receptor superfamily and acts as a receptor for the pleiotropic signaling molecule, leptin, which has roles in metabolism, reproduction, pregnancy, angiogenesis, growth and immunomodulation [59]. Previous reports describe hMSC *LEP* expression modulation in hypoxic conditions with alternate outcomes [23,25]. To our knowledge, we are the first to report BMhMSC *LEPR* upregulation in a reduced oxygen environment. The precise mechanistic consequence of *LEPR* upregulation in $G_HS_LO_{2\%}$ BMhMSC remains to be determined and it cannot be excluded at this point that the upregulation of LEPR may be as a consequence of the reduced serum (S_L) utilized in the optimized culture milieu.

Ontological examination of the 80 transcript BMhMSC fingerprint revealed a strong over-representation in specific areas complemented by significant CCRI KEGG pathway association (Supplementary Tables 5, 6, 7 & 8). These

findings highlighted downregulation of both *BMP2* and *IL8* receptor (*CXCR2*) signaling pathways as relevant to optimized BMhMSC recovery and mechanistic understanding. The importance of oxygen control was readily apparent by the increased associations with CCRI and significant association with TGFβ signaling for $O_{2\%}$ WS above that noted for $O_{2\%}$ only (Figure 6). The base media, GHSL, was consistent in this instance allowing clear dislocation and emphasis to be placed on the importance of controlled oxygen measures on BMhMSC biology. *CXCL1* (*GROα*) and *CXCL6* (*GCP-2*), both agonists for *CXCR2*, are pro-inflammatory cytokines with apparent roles in neutrophil attraction, angiogenic induction, stem cell self-renewal (*GROα*) and the transition of cells from suspension to substrate adherence through integrin activation [60–63]. The mechanistic role of *CXCL1* and *CXCL6* repression is unclear at this time and it cannot be excluded that this is a consequence of S_L versus S_H as opposed to the use of a reduced oxygen environment. Hypoxic repression of *BMP2* has been reported previously but is predominantly associated with repression of osteogenic differentiation of hMSC [56,64]. A single report details an association of reduced *BMP2* expression with enhanced hMSC isolation from the placental decidua basalis [65]. Here, we have demonstrated that BMP2 protein inhibits BMhMSC recovery in $G_HS_LO_{2\%}$ and provide clear lines of evidence supportive of a role for oxygen signaling in its repression.

The main driver behind optimization of hMSC recovery conditions is to accelerate robust cell isolation from bone marrow for procedural translation into GMP conditions and autologous cell therapy application. Current reports indicate that virtually all hMSC isolated in 20% O_2 die 4 days post-transplantation into ischemic heart models; this is suggested to be less severe in other transplant models (e.g., intravertebral disc, and osteoarthritis) [6]. It is therefore essential that isolation models are enhanced to promote prolonged hMSC survival post-transplant. Hypoxic preconditioned cells have attracted strong interest as a method to enhance hMSC survival post-transplant, although insufficient data are reported from human-derived cells to draw strong conclusions. However, rat MSC displayed reduced apoptosis, increased expression of pro-survival, pro-angiogenic and pro-differentiation factors when hypoxic preconditioned were kept for 24 h at 0.5% O_2, while hMSC exposed to 1% O_2 for 48 h displayed enhanced engraftment in a chick embryo model with apparent contribution to heart and brain tissues [28,66]. Enhanced BMhMSC isolation through combined $G_HS_LO_{2\%}$ and workstation control may in turn greatly improve post-transplant survival and the efficacy of stem cell therapies. However, it is important to note that all experimentation described in this manuscript was performed with BMhMSC isolated from the iliac crest of young, healthy, donors. It remains to be determined if the biology we have described above is consistent with bone marrow sourced from alternate sites or from an aged patient demographic.

Conclusion

The continued development of regenerative medicine coupled with increased applications will benefit from a stringent optimization phase of cell isolation and culture. In this report we have detailed a potential milestone in this process with the demonstration of a refinement in the hMSC isolation process. Engineered oxygen control and appropriate serum levels are essential to this process and appear to be coupled to distinct molecular alterations linked through *BMP2* and, putatively, *CXCR2*-signaling repression and autocrine upregulation of *LEPR* associated pathways.

Future perspective

The utility of adult, noncord-derived, hMSC in regenerative medicine-linked therapeutics is likely to diverge into two primary branches over the coming decade; autologous and allogeneic. The allogeneic branch will reflect the pull of industry toward an 'off-the-shelf' product and will become heavily reliant on established protocols for cell types with increased proliferative capacity sufficient to bank and distribute, in other words, MAPC. The autologous branch on the other hand will evolve toward a hospital-based production and delivery model where bespoke facilities at site will enable localized, individual, solutions. In the autologous model optimized isolation procedures are crucial

Executive summary

- Bone marrow-derived mesenchymal stem cell isolation is enhanced under controlled physiological oxygen and reduced serum conditions.
- Routine conditions for bone marrow-derived mesenchymal stem cell isolation incorporating ambient air and 10% serum result in reduced cell yields.
- Repeated opening of multiuser trigas incubator can result in reduced bone marrow-derived mesenchymal stem cell yields in comparison to workstation or single-use trigas incubators.
- Minimal and transient exposure to ambient air during isolation can significantly reduce cell numbers and impact on transcriptional profiles.
- Downregulation of BMP2 and TGFβ-linked signaling is associated with increased CFU-F and cell isolation.

to maximize the likelihood of success and to permit banking for repeat procedures where necessary. The coming decade will be accompanied by an increased awareness of the importance of parameter control during isolation and will be accompanied by an increased growth in new tool development to support optimized hMSC yield from individual patients.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

The authors wish to acknowledge the continued funding support from Keele University and the North Staffordshire Medical Institute. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet.* 3(4), 393–403 (1970).

•• **The first description of the isolation of what we now recognize to be mesenchymal stem cells.**

2 Pittenger MF, Mackay AM, Beck SC *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411), 143–147 (1999).

• **A robust description of the differentiation potential of human mesenchymal stem cells (hMSCs). This publication appeared a year after Thomson *et al.* described human embryonic stem cell (hESC) in the same journal and marks a key moment in the growth of the regenerative medicine field.**

3 Dominici M, Le Blanc K, Mueller I *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4), 315–317 (2006).

4 Griffiths SL, Griffiths D, Forsyth NR. Mesenchymal stem cell-based therapy for the older patient. *Aging Health* 7(3), 447–452 (2011).

5 ClinicalTrials www.clinicaltrials.gov

6 Das R, Jahr H, van Osch GJ, Farrell E. The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. *Tissue Eng. Part B Rev.* 16(2), 159–168 (2010).

7 Ezashi T, Das P, Roberts RM. Low O_2 tensions and the prevention of differentiation of hES cells. *Proc. Natl Acad. Sci. USA.* 102(13), 4783–4788 (2005).

•• **The first observation that reduced oxygen tensions play a beneficial role in the culture of hESCs by reducing that rate of spontaneous differentiation.**

8 Forsyth NR, Kay A, Hampson K, Downing A, Talbot R, McWhir J. Transcriptome alterations due to physiological normoxic (2% O2) culture of human embryonic stem cells. *Regen. Med.* 3(6), 817–833 (2008).

• **This observation follows on Ezashi *et al.*, and others, with the demonstration that widespread transcriptional alterations occur in hESCs as a direct result of reduced oxygen culture.**

9 Genbacev O, Zhou Y, Ludlow JW, Fisher SJ. Regulation of human placental development by oxygen tension. *Science* 277(5332), 1669–1672 (1997).

•• **A pivotal manuscript which describes the role of oxygen tension in trophoblast differentiation into cytotrophoblasts. Reduced oxygen tensions maintained an undifferentiated trophoblast population.**

10 Cipolleschi MG, Dello SP, Olivotto M. The role of hypoxia in the maintenance of hematopoietic stem cells. *Blood* 82(7), 2031–2037 (1993).

11 D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J. Cell Sci.* 117, 2971–2981 (2004).

12 Danet GH, Pan Y, Luongo JL, Bonnet DA, Simon MC. Expansion of human SCID-repopulating cells under hypoxic conditions. *J. Clin. Invest.* 112(1), 126–135 (2003).

13 Ivanovic Z, Dello SP, Trimoreau F, Faucher JL, Praloran V. Primitive human HPCs are better maintained and expanded *in vitro* at 1 percent oxygen than at 20 percent. *Transfusion* 40(12), 1482–1488 (2000).

• **An important observation describing the beneficial role that reduced oxygen culture conditions have on hematopoietic progenitor cells.**

14 Mazumdar J, O'Brien WT, Johnson RS *et al.* O_2 regulates stem cells through Wnt/beta-catenin signalling. *Nat. Cell Biol.* 12(10), 1007–1013 (2010).

15 Studer L, Csete M, Lee SH *et al.* Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J. Neurosci.* 20(19), 7377–7383 (2000).

• **An early description of the role of reduced oxygen in stem cell culture. In this example, neural stem cells were shown to display enhanced behavioral properties in a reduced oxygen setting.**

16 Forsyth NR, Musio A, Vezzoni P, Simpson AH, Noble BS, McWhir J. Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. *Cloning Stem Cells* 8(1), 16–23 (2006).

17 Forsyth NR, McWhir J. Human embryonic stem cell telomere length impacts directly on clonal progenitor isolation frequency. *Rejuvenation Res.* 11(1), 5–17 (2008).

18 Kumar D, Gupta S, Yang Y, Forsyth NR. αVβ5 and CD44 are oxygen-regulated human embryonic stem cell attachment factors. *Biomed. Res. Int.* 2013, 729281 (2013).

19 Lengner CJ, Gimelbrant AA, Erwin JA *et al.* Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. *Cell* 141(5), 872–883 (2010).

20 Ludwig TE, Levenstein ME, Jones JM *et al.* Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* 24(2), 185–187 (2006).

21 Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* 7(2), 150–161 (2010).

22 Adesida AB, Mulet-Sierra A, Jomha NM. Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells. *Stem Cell Res. Ther.* 3(2), 9 (2012).

23 Basciano L, Nemos C, Foliguet B *et al.* Long term culture of mesenchymal stem cells in hypoxia promotes a genetic program maintaining their undifferentiated and multipotent status. *BMC Cell Biol.* 12(12), doi:10.1186/1471-2121-12-12 (2011).

24 Fehrer C, Brunauer R, Laschober G *et al.* Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. *Aging Cell* 6(6), 745–757 (2007).

25 Fink T, Abildtrup L, Fogd K *et al.* Induction of adipocyte-like phenoType In human mesenchymal stem cells by hypoxia. *Stem Cells* 22(7), 1346–1355 (2004).

26 Grayson WL, Zhao F, Izadpanah R, Bunnell B, Ma T. Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J. Cell Physiol.* 207(2), 331–339 (2006).

27 Holzwarth C, Vaegler M, Gieseke F *et al.* Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells. *BMC Cell Biol.* 11, 11 (2010).

28 Hung SC, Pochampally RR, Hsu SC *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS. ONE* 2(5), e416 (2007).

29 Jin Y, Kato T, Furu M *et al.* Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. *Biochem. Biophys. Res. Commun.* 391(3), 1471–1476 (2010).

30 Pattappa G, Thorpe SD, Jegard NC, Heywood HK, de Bruijn JD, Lee DA. Continuous and uninterrupted oxygen tension influences the colony formation and oxidative metabolism of human mesenchymal stem cells. *Tissue Eng. Part C Methods* 19(1), 68–79 (2013).

31 Potier E, Ferreira E, Andriamanalijaona R *et al.* Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. *Bone* 40(4), 1078–1087 (2007).

32 Sengupta S, Park SH, Patel A, Carn J, Lee K, Kaplan DL. Hypoxia and amino acid supplementation synergistically promote the osteogenesis of human mesenchymal stem cells on silk protein scaffolds. *Tissue Eng. Part A* 16(12), 3623–3634 (2010).

33 Tamama K, Kawasaki H, Kerpedjieva SS, Guan J, Ganju RK, Sen CK. Differential roles of hypoxia inducible factor subunits in multipotential stromal cells under hypoxic condition. *J. Cell Biochem.* 112(3), 804–817 (2011).

34 Weil BR, Abarbanell AM, Herrmann JL, Wang Y, Meldrum DR. High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 296(6), R1735–R1743 (2009).

35 Carrancio S, Lopez-Holgado N, Sanchez-Guijo FM *et al.* Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. *Exp. Hematol.* 36(8), 1014–1021 (2008).

36 dos Santos F, Andrade PZ, Boura JS, Abecasis MM, da Silva CL, Cabral JM. *Ex vivo* expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. *J. Cell Physiol.* 223(1), 27–35 (2010).

37 Grayson WL, Zhao F, Bunnell B, Ma T. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem. Biophys. Res. Commun.* 358(3), 948–953 (2007).

38 Rosova I, Dao M, Capoccia B, Link D, Nolta JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* 26(8), 2173–2182 (2008).

39 Agata H, Watanabe N, Ishii Y *et al.* Feasibility and efficacy of bone tissue engineering using human bone marrow stromal cells cultivated in serum-free conditions. *Biochem. Biophys. Res. Commun.* 382(2), 353–358 (2009).

40 Bieback K, Hecker A, Kocaömer A *et al.* Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells* 27(9), 2331–2341 (2009).

41 Meuleman N, Tondreau T, Delforge A *et al.* Human marrow mesenchymal stem cell culture: serum-free medium allows better expansion than classical alpha-MEM medium. *Eur. J. Haematol.* 76(4), 309–316 (2006).

42 Pérez-Ilzarbe M, Díez-Campelo M, Aranda P *et al.* Comparison of *ex vivo* expansion culture conditions of mesenchymal stem cells for human cell therapy. *Transfusion* 49(9), 1901–1910 (2009).

43 Funes JM, Quintero M, Henderson S *et al.* Transformation of human mesenchymal stem cells increases their dependency on oxidative phosphorylation for energy production. *Proc. Natl Acad. Sci. USA* 104(15), 6223–6228 (2007).

44 Li YM, Schilling T, Benisch P *et al.* Effects of high glucose on mesenchymal stem cell proliferation and differentiation. *Biochem. Biophys. Res. Commun.* 363(1), 209–215 (2007).

45 Mylotte LA, Duffy AM, Murphy M *et al.* Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. *Stem Cells* 26(5), 1325–1336 (2008).

46 Deschepper M, Oudina K, David B *et al.* Survival and function of mesenchymal stem cells (MSCs) depend on glucose to overcome exposure to long-term, severe and continuous hypoxia. *J. Cell Mol. Med.* 15(7), 1505–1514 (2011).

47 Kao GS, Kim HT, Daley H *et al.* Validation of short-term handling and storage conditions for marrow and peripheral blood stem cell products. *Transfusion* 51(1), 137–147 (2011)

48 Wimpenny I, Hampson K, Yang Y, Ashammakhi N, Forsyth NR. One-step recovery of marrow stromal cells on nanofibers. *Tissue Eng. Part C. Methods* 16(3), 503–509 (2010).

49 Justesen J, Stenderup K, Eriksen EF, Kassem M. Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures. *Calcif. Tissue Int.* 71(1), 36–44 (2002).

50 da Huang W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 37(1), 1–13 (2009a).

51 da Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4(1), 44–57 (2009b).

52 Ohnishi S, Yasuda T, Kitamura S, Nagaya N. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. *Stem Cells* 25(5), 1166–1177 (2007).

53 Westfall SD, Sachdev S, Das P *et al.* Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. *Stem Cells Dev.* 17(5), 869–881 (2008).

54 Martin-Rendon E, Hale SJ, Ryan D *et al.* Transcriptional profiling of human cord blood CD133+ and cultured bone marrow mesenchymal stem cells in response to hypoxia. *Stem Cells* 25(4), 1003–1012 (2007).

55 Zhambalova AP, Darevskaya AN, Kabaeva NV, Romanov YA, Buravkova LB. Specific interaction of cultured human mesenchymal and hemopoietic stem cells under conditions of reduced oxygen content. *Bull. Exp. Biol. Med.* 147(4), 525–530 (2009).

56 Salim A, Nacamuli RP, Morgan EF, Giaccia AJ, Longaker MT. Transient changes in oxygen tension inhibit osteogenic differentiation and Runx2 expression in osteoblasts. *J. Biol. Chem.* 279(38), 40007–40016 (2004).

57 Felka T, Schafer R, Schewe B, Benz K, Aicher WK. Hypoxia reduces the inhibitory effect of IL-1beta on chondrogenic differentiation of FCS-free expanded MSC. *Osteoarthritis Cartilage* 17(10), 1368–1376 (2009).

58 Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. *Cell Transplant.* 19(1), 29–42 (2010).

59 Perez-Perez A, Maymo J, Gambino Y *et al.* Leptin stimulates protein synthesis-activating translation machinery in human trophoblastic cells. *Biol. Reprod.* 81(5), 826–832 (2009).

60 Catusse J, Liotard A, Loillier B, Pruneau D, Paquet JL. Characterization of the molecular interactions of interleukin-8 (CXCL8), growth related oncogen alpha (CXCL1) and a non-peptide antagonist (SB 225002) with the human CXCR2. *Biochem. Pharmacol.* 65(5), 813–821 (2003).

61 Hannelien V, Karel G, Jo VD, Sofie S. The role of CXC chemokines in the transition of chronic inflammation to esophageal and gastric cancer. *Biochim. Biophys. Acta* 1825(1), 117–129 (2012).

62 Krtolica A, Larocque N, Genbacev O *et al.* GROalpha regulates human embryonic stem cell self-renewal or adoption of a neuronal fate. *Differentiation* 81(4), 222–232 (2011).

63 Wuyts A, Proost P, Lenaerts JP, Ben-Baruch A, Van DJ, Wang JM. Differential usage of the CXC chemokine receptors 1 and 2 by interleukin-8, granulocyte chemotactic protein-2 and epithelial-cell-derived neutrophil attractant-78. *Eur. J. Biochem.* 255(1), 67–73 (1998).

64 Yang DC, Yang MH, Tsai CC, Huang TF, Chen YH, Hung SC. Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. *PLoS One* 6(9), e23965 (2011).

65 Huang YC, Yang ZM, Jiang NG *et al.* Characterization of MSCs from human placental decidua basalis in hypoxia and serum deprivation. *Cell Biol. Int.* 34(3), 237–243 (2010).

66 Chacko SM, Ahmed S, Selvendiran K, Kuppusamy ML, Khan M, Kuppusamy P. Hypoxic preconditioning induces the expression of prosurvival and proangiogenic markers in mesenchymal stem cells. *Am. J. Physiol. Cell. Physiol.* 299(6), C1562–C1570 (2010).