

Proteomic analysis of the impact of static culturing on the expansion of rat bone marrow mesenchymal stem cells

Stefan Peter · Caroline Evans · Saw Y. Ow ·
Andy M. Scutt · Phillip C. Wright ·
Catherine A. Biggs

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Abstract The clinical potential of mesenchymal stem cells (MSC) in tissue engineering and regenerative medicine is due to their self-renewal, proliferation and multi-lineage differentiation potential. Clinical use requires large cell numbers; which can, theoretically, be generated by ex vivo expansion of plastic adherent, MSC subpopulation, of bone marrow cells (BMC). Effects of serial culture on MSC phenotype were investigated using non-gel based quantitative proteomic methodology for static monolayer cultures of rat BMC. In total, 382 proteins were relatively quantified (≥ 2 peptides). Nine proteins were

up-regulated and seven down-regulated at passage 4 relative to passage 2 ($p \leq 0.05$). We propose that serial culture impacts on MSC expansion (observed decline in colony forming potential and colony size) is through a combination of osteogenic differentiation and ageing/senescence and propose six novel protein biomarkers as candidates for quality control purposes in bioprocessing.

Keywords Ageing · iTRAQ · Mesenchymal stem cells · Proteomics · Static cultures

Stefan Peter and Caroline Evans contributed equally to the study.

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S. Peter
Academic Urology Unit and Institute for Cancer Studies,
Medical School, The University of Sheffield,
Beech Hill Road, Sheffield S10 2RX, UK
e-mail: stefan.peter@sheffield.ac.uk

C. Evans · S. Y. Ow · P. C. Wright · C. A. Biggs (✉)
ChELSI Institute, Department of Chemical and Biological
Engineering, The University of Sheffield,
Mappin Street, Sheffield S1 3JD, UK
e-mail: c.biggs@sheffield.ac.uk

C. Evans
e-mail: caroline.evans@sheffield.ac.uk

Introduction

Mesenchymal stem cells (MSC) are the focus of much research interest due to their potential therapeutic use,

S. Y. Ow
e-mail: s.ow@sheffield.ac.uk

P. C. Wright
e-mail: p.c.wright@sheffield.ac.uk

A. M. Scutt
Department of Human Metabolism, Medical School,
The University of Sheffield, Beech Hill Road,
Sheffield S10 2RX, UK
e-mail: a.m.scutt@sheffield.ac.uk

based on their multi-lineage differentiation potential (they can give rise to myocytes, chondrocytes, adipocytes, and osteogenic cells), and their self-renewal and proliferation capacities, which allow easy *ex vivo* expansion (Pittenger et al. 1999).

Proteomic techniques provide methods to identify the characteristic protein profiles of MSC populations (Dominici et al. 2006). Thus far, much of MSC proteomic research has used two-dimensional gel electrophoresis (2DE) to quantify and identify changes in the MSCs proteome occurring during serial culture (Sun et al. 2006; Celebi and Elcin 2009), during differentiation, in particular along osteogenic and adipogenic lineages (Lee et al. 2006; Zhang et al. 2007) and to analyse a range of stem cell, MSC and differentiation markers (Park et al. 2007). Although 2DE has yielded useful data, this technique has major limitations since not all proteins can be visualised in this way, particularly large and/or hydrophobic proteins (Maurer and Kuschinsky 2007).

An alternative non-gel-based quantitative MS approach to 2DE is the technique of an isobaric tag for relative and absolute quantitation (iTRAQ). iTRAQ allows simultaneous protein identification and relative quantification in a multiplex format to provide more accurate relative quantification and improved sequence coverage of proteins compared to 2DE (Zhang et al. 2005). All proteins analysed in an iTRAQ study are identified rather a specific subset that is typically analysed by 2DE. An overview of iTRAQ as a proteomics tool and its scope is available elsewhere (Noirel et al. 2011).

This study investigates the impact of serial culture on the expansion of MSC in static cultures systems by analysing the change in protein expression profiles using a high throughput iTRAQ-based proteomic workflow.

Materials and methods

Isolation of rat bone marrow cells (BMC)

Rat BMCs were used rather than human cells due to their reproducibility, ready availability and minimal risk of infection. The BMCs were obtained from the femurs and tibias of Wistar Rats (ages 2–4 months) by flushing out the bone marrow under sterile conditions as previously described (Scutt et al. 2003).

Mesenchymal stem cells (MSC) static monolayer culture

Ten milliliters freshly harvest BMCs were suspended in 10 ml culture medium [DMEM 10 % v/v foetal calf serum (FCS), 2 mM Glutamax and 2 mM penicillin/streptomycin] and seeded into a T75 flask, at 10^6 cells/ml. The cells were incubated at 37 °C in 7.5 % CO₂ and incubated for 4 passages. The first medium change was after 5 days and thereafter twice weekly until ~90 % confluent. Cells were detached using 0.25 % trypsin/1 mM EDTA solution. The detached cells were split 1:2 into new sterile T75-flasks. At passage 2 and 4, cells from 3 confluent flasks were collected for proteomic analysis.

Fibroblastic colony forming unit (CFU-f) and osteogenic differentiation assays

All CFU-f cultures and osteogenic differentiation assays were conducted in triplicate with either 0.3 ml freshly harvested BMCs (passage number 0) or 4×10^3 MSCs (obtained from monolayer cultures after trypsinising) as described by Scutt et al. (2003).

The proteomic workflow using iTRAQ can be found in Fig. 1, and the specific steps are outlined below.

Protein extraction

Pooled and pelleted cells from passage 2 and 4 were re-suspended in 300 µl PBS prior to disruption by probe sonication using the Sonifier 450 (Branson). Soluble proteins were recovered by centrifugation at $21,000 \times g$ for 30 min at 4 °C and protein assayed (Glen et al. 2008). Two aliquots of 100 µg of protein from both conditions (cells harvested at passage 2 and 4) were acetone precipitated in preparation for iTRAQ labelling.

Protein digestion and iTRAQ labelling

Peptides were generated by proteolytic digestion using trypsin, following protein reduction, cysteine blocking. These steps and iTRAQ labelling were carried out using the iTRAQ 4-plex kit (Applied Biosystems–now ABSciex) and performed following the manufacturer's instructions. The two replicate samples from passage 2 were labelled with reagents 114 and 115,

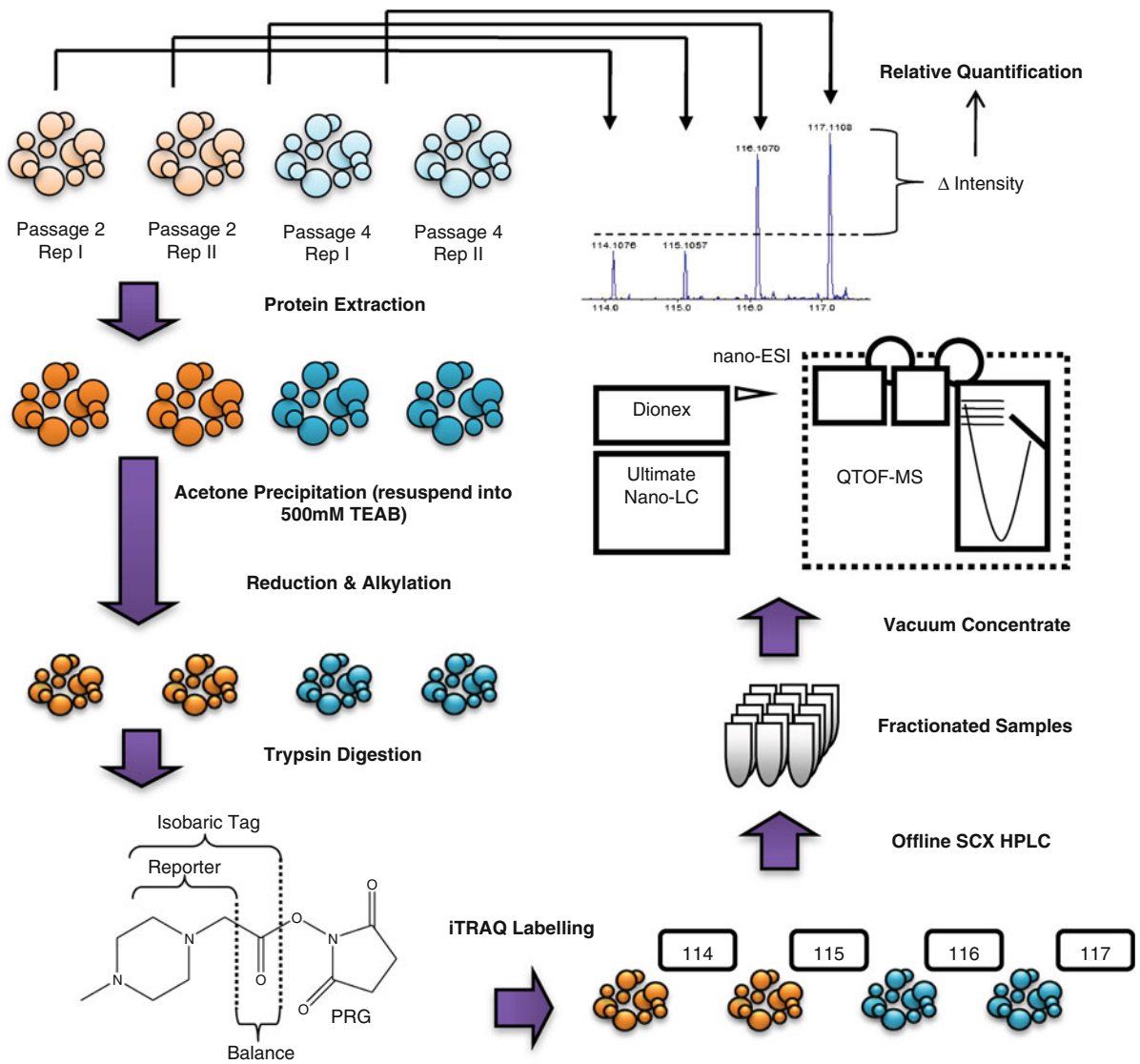


Fig. 1 Schematic iTRAQ workflow showing protein extraction, trypsin digest, iTRAQ labelling, peptide fractionation and mass spectrometry

and the samples from passage 4 were labelled with the reagents 116 and 117. Afterwards, the labelled samples were combined and vacuum concentrated, fractionated using strong cation exchange and analysed by LC–MS/MS using a QSTAR XL Hybrid ESI Quadrupole time of flight tandem mass spectrometer (Applied Biosystems, Framingham, MA; MDS-Sciex, Concord, Ontario, Canada) coupled with an online capillary liquid chromatography system (Famos, Swichos and ultimate from Dionex/LC Packings, Amsterdam, The Netherlands) as described by Glen et al. (2008).

Protein identification and relative quantification

Protein Pilot software version 2.0 (Applied Biosystems, MDS-Sciex) was used for protein quantification and identification as reported previously (Glen et al. 2008). Identifications were made using rat NCBIr protein database (36,496 entries, downloaded June 2008). Only proteins identified with at least 95 % confidence (ProtScore of 1.3) were reported. Peptides with ≥ 80 % confidence were used for protein identification. A concatenated target-decoy database search strategy was also employed to estimate the rate of false

positives (Boehm et al. 2007), which was calculated to be within the 5 % recommended for reporting proteomic data. Quantification data were parsed into Mathematica version 7.0 (Wolfram, UK). Protein quantifications were obtained by computing the geometric means of the reporters' intensities. Data was isotope corrected, and bias normalization performed correcting the median ratio of each comparison toward unity.

Functional annotation of proteins

The Panther (Protein analysis through evolutionary relationships) classification system was used to assign molecular function and biological process of proteins. The functional relationship of differentially expressed proteins was further investigated using MetaCore™ (GeneGo Inc.) software.

Statistical analysis

Data for cell cultures and CFU-f assays are expressed as mean values \pm standard deviation. Each culture and assay was performed in triplicate. Statistical significance was determined by Student's *t* test, with <0.05 considered significant. Changes in protein expression between passage 2 and 4 were qualified using a *t* test algorithm developed in house (Pham et al. 2010).

Results

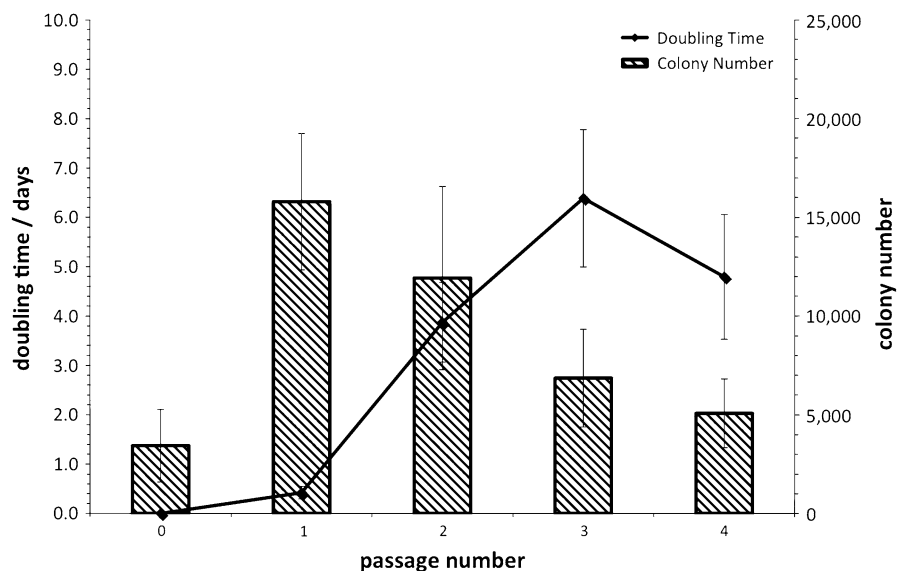
Morphology and proliferation rate of bone marrow derived MSC

MSCs were expanded in static monolayer cultures by serial passage. Viable cell number, CFU-f and morphology were assessed over 4 passages. From an initial seeding of 4×10^3 adherent cells for Passage 0, the total viable cell number increased continually to 6×10^7 after 4 passages, which represents a 15,000-fold expansion. The cell viability was $>99\%$ for the whole culture period. The time to confluence was reduced for passages 1 to passage 4 inclusive, compared to passage 0, consistent with the finding of Celebi and Elcin (2009) when also analysing rat bone marrow derived MSC. The proliferation rate of MSCs, measured in doubling time, decreased after passage 1 but did not change significantly between passage 2 and 4 (Fig. 2).

Colony forming potential after each passage is also shown in Fig. 2. An initial increase in colony forming potential observed for passage 1 relative to passage 0 (reflecting enrichment for MSC by adherence to the plastic tissue culture flask following plating out freshly isolated cells) was followed by a decrease in CFU-f over successive passages.

The average colony size also decreased after serial sub-culturing. MSCs expanded in static cultures for 2

Fig. 2 Proliferation rate (doubling time) and colony development (CFU-f) for rat bone marrow derived MSCs with increasing passage number. Cells were expanded in static cultures with standard DMEM. Plating density was $\sim 10^6$ cells per T-75 flask. Data are presented as mean \pm SD from three different samples



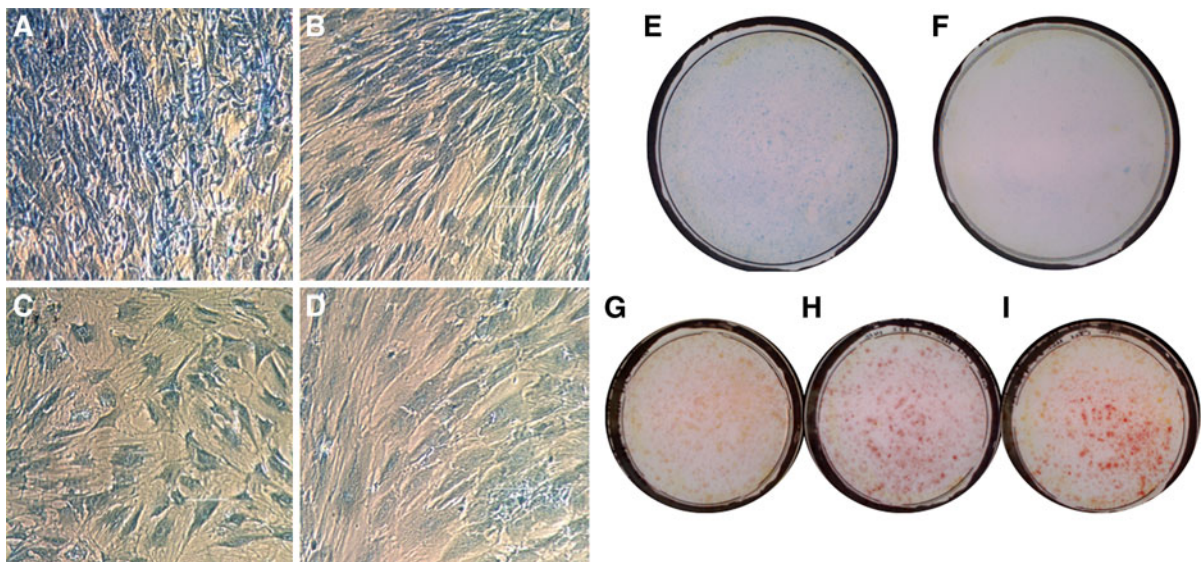


Fig. 3 Results from the methylene blue and osteogenic differentiation assays Methylene blue-stained adherent rat bone marrow cells in the primary culture at **a** passage level 0, **b** passage level 2, **c** passage level 3 and **d** passage level 4. The four pictures were taken with $\sim 100\times$ magnification. Methylene blue-stained MSCs

passages formed small but recognisable colonies during the CFU-f assay. The colonies formed by MSCs passaged more than twice were barely visible on the plates (Fig. 3). Interestingly, the morphology of the MSC in the monolayer cultures changed over time as the cells became wider and larger (Fig. 3). Osteogenic differentiation potential of passaged MSCs was confirmed by assaying for the presence of alkaline phosphatase, calcium and collagen (Fig. 3), which are biochemical markers of osteoblast differentiation (Scutt et al. 2003).

Proteomic changes of altered MSC phenotype during culture

The proteomic changes underlying the observed changes in MSC phenotype during serial culture in static culture systems were investigated using an iTRAQ-based workflow. From the iTRAQ data, 23,231 mass spectra were acquired which were identified as 7,134 distinct peptide sequences. A total of 382 proteins were identified by ≥ 2 peptides. The full peptide and protein lists are shown in Supplementary Table 1. In order to calculate the relative expression levels of the proteins at passage 2 and 4, the mean \log_{10} ratio of the peptides labelled from both replicates

as CFU-f cultures with colony formation at **e** passage level 2 and **f** passage level 4. CFU-f cultures used for osteogenic differentiation assay were stained with **g** naphthol phosphate and Fast red for alkaline phosphatase, **h** Alizarin red for calcium and **i** Sirius red for collagen (I)

at passage 2 (114:114, 115:114) were compared to the mean of the \log_{10} ratios of the peptides labelled from passage 4 (116:114) and (117:114). A total of 16 proteins were differentially expressed with 9 proteins up-regulated and 7 down-regulated (p value 0.05, Bonferroni multiple test correction applied). The lists of the up-regulated proteins and down-regulated protein are shown in Table 1 with their functional annotation (Huang et al. 2008, 2009).

Discussion

The effect of serial subculturing from static cultures on the proteome of rat bone marrow derived MSCs has been determined: whilst the overall proliferation rate of MSCs only decreased slightly over 4 passages (Fig. 2), the effects on CFU-f potential (Fig. 2) and the morphology of the MSC cultures (Fig. 3) were more marked. After 4 passages, a change in the cells morphology can be observed as they became wider, which together with decreased colony forming ability, can indicate ageing of the culture (Stolzing and Scutt 2006).

The proteome of MSCs from passage 2 was compared to that of MSCs from passage 4, to

Table 1 Proteins up- and down-regulated in Passage 4 (P4) compared to Passage 2 (P2)

ID	Gene Name	Peptide #	P4:P2	Molecular functions	Biological processes
Up-regulated					
Actn4	Actinin alpha 4	74	1.6	Cytoskeletal protein, actin binding cytoskeletal protein, non-motor actin binding	Cell structure and motility, cell structure
Cnn1	Calponin 1, basic, smooth muscle	13	2.7	Cytoskeletal protein, actin binding cytoskeletal protein, non-motor actin binding	Intracellular protein traffic, developmental processes, mesoderm development, cell structure and motility
Tpi1	Triosephosphate isomerase	40	1.9	Isomerase	Muscle contraction
Ppia	Peptidylprolyl isomerase A (cyclophilin A)	113	2.5	Isomerase, other isomerase	Signal transduction, intracellular signalling cascade, cell cycle, cell cycle control
Nutf2	Nuclear transport factor 2	2	2.9	Miscellaneous function, other miscellaneous function	Intracellular protein traffic, nuclear transport, protein targeting and localization, protein targeting
Plod2	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	87	3.2	Oxidoreductase, oxygenase	Intracellular protein traffic, nuclear transport
IQGAP1	IQ motif containing GTPase activating protein 1	21	1.6	Select regulatory molecule, G-protein modulator, other G-protein modulator	Protein metabolism and modification, other protein metabolism
Kpna1	Karyopherin (importin) alpha 1	3	1.7	Transfer/carrier protein	Protein metabolism and modification, protein folding, intracellular protein traffic, nuclear transport, immunity and defense
Anxa2	Annexin A2	61	1.8	Transfer/carrier protein, select calcium binding protein, annexin	Carbohydrate metabolism, glycolysis, other metabolism
Down-regulated					
Crsp1	Cysteine and glycine-rich protein 1	10	-2.3	Cytoskeletal protein, actin binding cytoskeletal protein	Transport, ion transport, anion transport, homeostasis, other homeostasis activities
Clic4	Chloride intracellular channel 4 (mitochondrial)	28	-1.3	Ion channel, anion channel	Immunity and defense, developmental processes, other developmental process
Pgam1	Phosphoglycerate mutase 1	15	-2.2	Isomerase, mutase	Protein metabolism and modification, protein biosynthesis
AHNAK	AHNAK nucleoprotein	48	-2.7	Nucleic acid binding, binding, protein binding	Protein metabolism and modification, amino acid activation
Rps27a	Ribosomal protein S27a	59	-2.0	Nucleic acid binding, ribosomal protein	Carbohydrate metabolism, glycolysis
Eif5a	Eukaryotic translation initiation factor 5A	34	-1.6	Nucleic acid binding, translation factor, translation initiation factor	Protein metabolism and modification, proteolysis
Eprs	Glutamyl-prolyl-tRNA synthetase	11	-2.2	Synthase and synthetase, synthetase, aminoacyl-tRNA synthetase	Developmental processes

Proteins in *bold* are novel findings for differentially regulated proteins in mesenchymal stem cells

determine alterations in protein expression associated with serial culturing and loss of expansion potential. Cells from passage 2 were chosen in preference to passage 1 in order to allow the MSC to adapt to in vitro conditions and adopt a stable phenotype. Previous reports had suggested that differentiation and proliferation capacity remained static from passage 2 to 10 (Bruder et al. 1997). However, the CFU-f assay data showed a dramatic decrease in colony numbers at passage 4, and because of this, passages 2 and 4 were chosen for comparison.

In general, there is agreement on proteins involved in serial culturing, ageing and osteogenic differentiation (Table 1) in our study compare to other MSC studies (Sun et al. 2006; Celebi and Elcin 2009; Kim et al. 2008). For example, the up-regulation of Annexin A2 at passage 4 relative to passage 2 has also been demonstrated in human bone marrow derived MSC during serial culturing. The glycolytic enzymes enolase and pyruvate kinase were also shown to be up-regulated in human BMC derived MSC (Sun et al. 2006). Whilst this current study did not identify these particular enzymes, a related enzyme on the same metabolic pathway, triose phosphate isomerase was up-regulated (Table 1).

The up-regulation of the proteins nuclear transport factor 2 and karyopherin (also known as importin) at passage 4 compared to passage 2 represent novel findings—these are components of the RAN transport pathway (Table 1). In terms of functional significance, the RAN transport pathway acts as a molecular switch for the karyopherin to regulate nuclear transport during cell cycle, interphase cells (Yudin and Fainzilber 2009). Another 4 other proteins that were down-regulated: the cysteine and glycine-rich protein 1, AHNAK nucleoprotein, ribosomal protein S27 and glutamyl-prolyl-tRNA synthetase are also novel protein changes associated with serial culture (Table 1).

The list of proteins that were up-regulated and down-regulated between passages 2 and 4 were further analysed using the Metacore platform (GeneGo Inc., St. Joseph, MI). Metacore provides an interface for submission of proteins in terms of gene identity for searching against an extensive, manually curated database of proteins involved in biological networks associated with transcriptional and metabolic regulation, and in cell signalling incorporating published protein–protein, protein–DNA interactions (www.genego.com). The network analysis identified 7

networks (regulatory and metabolic), of which the 3 most highly significant (5 or more root nodes, Z score of >40) involved the protein components of

1. Ubiquitin (Rps27a), alpha actinin 4, nuclear transport factor 2, karyopherin alpha 1,
2. Ubiquitin (Rps27a), IQGAP1, karyopherin alpha 1, AHNAK,
3. Annexin A2, cyclophilin A, AHNAK and calponin 1.

The top 3 regulatory networks were RAN transport (as mentioned above) cell cycle and glycolysis. Of note, the glycolytic enzymes, triose phosphate isomerase and phosphoglycerate mutase were found to be altered in this study, suggesting alterations in energy metabolism, consistent with other studies (Sun et al. 2006).

In summary, the utility of the iTRAQ workflow has been demonstrated as a useful tool to investigate the change in phenotype of MSC during serial culture, and has generated results that are both novel and complementary to previous 2DE proteomic studies on MSCs. Crucially, the data highlights the impact of the culture condition of the MSC with significant loss of self-renewal capacity with repeated serial subculture. Taking all these findings together, the observed reduction in CFU-f in this study could be explained by a combination of osteogenic differentiation and with signs of potential ageing of cells in the culture.

The use of iTRAQ to identify key up- and down-regulated proteins related to the loss of self-renewal capacity during culture expansion, could contribute to the development of a panel of biomarkers for monitoring cell status. Such a panel could in turn be used as a monitor of quality control, assessing the validity of the quality of large scale MSC preparations intended for tissue engineering purposes.

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