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# Multiparameter Analysis of Human Bone Marrow Stromal Cells Identifies Distinct Immunomodulatory and Differentiation-Competent Subtypes

Sally James,[1,5] James Fox,[1,5] Farinaz Afsari,[1] Jennifer Lee,[1] Sally Clough,[1] Charlotte Knight,[1] James Ashmore,[1] Peter Ashton,[1] Olivier Preham,[1] Martin Hoogduijn,[2] Raquel De Almeida Rocha Ponzoni,[3] Y. Hancock,[3,4] Mark Coles,[1] and Paul Genever[1,*]

[1]Department of Biology, University of York, York YO10 5DD, UK
[2]Erasmus Medical Centre, Dr. Molewaterplein 50, Rotterdam 3015 GE, the Netherlands
[3]Department of Physics, University of York, York YO10 5DD, UK
[4]York Centre for Complex Systems Analysis, University of York, York YO10 5GE, UK
[5]Co-first author
*Correspondence: paul.genever@york.ac.uk
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## SUMMARY

Bone marrow stromal cells (BMSCs, also called bone-marrow-derived mesenchymal stromal cells) provide hematopoietic support and immunoregulation and contain a stem cell fraction capable of skeletogenic differentiation. We used immortalized human BMSC clonal lines for multi-level analysis of functional markers for BMSC subsets. All clones expressed typical BMSC cell-surface antigens; however, clones with trilineage differentiation capacity exhibited enhanced vascular interaction gene sets, whereas non-differentiating clones were uniquely CD317 positive with significantly enriched immunomodulatory transcriptional networks and high IL-7 production. IL-7 lineage tracing and CD317 immunolocalization confirmed the existence of a rare non-differentiating BMSC subtype, distinct from *Cxcl12*-DsRed$^{+}$ perivascular stromal cells in vivo. Colony-forming CD317$^{+}$ IL-7$^{hi}$ cells, identified at ~1%–3% frequency in heterogeneous human BMSC fractions, were found to have the same biomolecular profile as non-differentiating BMSC clones using Raman spectroscopy. Distinct functional identities can be assigned to BMSC subpopulations, which are likely to have specific roles in immune control, lymphopoiesis, and bone homeostasis.

## INTRODUCTION

Bone marrow stromal cells (BMSCs, also called bone-marrow-derived mesenchymal stromal cells) are heterogeneous populations that likely contain varying levels of tripotent (osteogenic, adipogenic, and chondrogenic [OAC]) stem-cell-like cells; cells with restricted potency (bi-, uni-, and nullipotent), committed precursors, and other stromal cell types. Phenotypic variations probably reflect in vivo functional diversity and a biological requirement for distinct stromal subsets with specific roles in bone marrow maintenance. Single-cell-derived BMSC clone analysis has identified considerable variation in differentiation capacity, ranging from OAC tripotency to nullipotency in vitro (Muraglia et al., 2000; Okamoto et al., 2002; Russell et al., 2010, 2011) and in vivo (Kuznetsov et al., 1997), which may indicate the existence of BMSC subtypes with varied potencies and/or a hierarchical developmental progression. BMSCs also possess significant immunomodulatory characteristics and can influence all aspects of immune cell function via cell-cell interaction and immunoregulatory factor secretion (Nauta and Fibbe, 2007), although clear demarcation between the skeletogenic and immunomodulatory capacity of BMSCs has not been made. Identification of human BMSCs often relies on non-discriminatory epitope detection (such as CD29, CD44, CD73, CD90 [THY-1], CD105, CD106 [VCAM-1], and CD166) with lack of hematopoietic markers (such as CD34, CD14, and CD45) (Dominici et al., 2006). Additional BMSC surface antigens have been described, including STRO-1 (Simmons and Torok-Storb, 1991), CD146 (MCAM) (Sacchetti et al., 2007), CD271 (LNGFR) (Quirici et al., 2002), Nestin (Méndez-Ferrer et al., 2010), platelet-derived growth factor receptor alpha (PDGFRα)/CD51 (Pinho et al., 2013), LNGFR$^{+}$THY-1$^{+}$VCAM-1$^{hi+}$ (Mabuchi et al., 2013), and in mice, leptin receptor (LepR/CD295) (Zhou et al., 2014). However, cell-sorting experiments using these markers all show that they contain the colony-forming, differentiation-competent BMSC population, demonstrating that other, as yet undefined BMSC subtypes exist and further resolution of BMSC heterogeneity is required. In vitro studies of BMSC functionality are hindered by their limited lifespan as replicative senescence occurs during culture, thus limiting the number and depth of studies that can be performed. To address these issues, we immortalized human BMSCs using human telomerase reverse transcriptase (hTERT), followed by clonal isolation to generate a panel of BMSC lines (hTERT-BMSCs). This strategy enabled in-depth, multiparameter analysis of variation in human BMSC subpopulations with different behavioral traits that subsequently could be examined in heterogeneous primary BMSCs. We unveil a range of biophysico-chemical markers

for identification of different BMSC subsets with specific immunomodulatory and differentiation competencies.

## RESULTS

### Generation and Phenotyping of Variant hTERT Immortalized Clonal Cell Lines from Primary BMSCs

A heterogeneous population of primary bone marrow BMSCs was isolated from one donor (FH181) based on their strong growth properties and multilineage differentiation potential. A single donor was deliberately selected to guard against expected genetic/lifestyle factors that cause inter-donor variation and would cloud interpretation of BMSC sub-population variation. A lentiviral expression system was used to overexpress hTERT in these cells, and stable-expressing lines were generated from single-cell-derived colonies. From numerous hTERT-BMSCs generated, our attention focused initially on four clonal lines selected for their strong clonal and stable growth characteristics (termed Y101, Y102, Y201, and Y202) that were maintained for over 400 days, showing exponential growth (Figure S1A) and elevated telomerase activity (Figure S1B). Their BMSC marker profile was determined by flow cytometry; all were positive for CD29, CD44, CD73, CD90, CD105, and CD166 and negative for CD34 and CD45 (Figure 1A), a profile matching their parental cells and typical of human primary BMSCs. All clones were also negative for CD11b, CD14, CD169, CD1a, CD4, CD11c, and CD83 (data not shown). It has been suggested previously that some BMSC lines may undergo oncogenic transformation either through hTERT transduction or extended time in culture (Serakinci et al., 2004). We found no evidence of tumorigenicity in our hTERT-BMSCs using in vitro colony transformation assays for non-adherent growth (Figure S1C) or following subcutaneous injection into immunocompromised mice (data not shown). Distinct differences were observed in individual cell morphologies and single-cell-derived colonies: Y101 and Y201 shared characteristics that were distinct from Y102 and Y202 lines. Y101 and Y201 lines had elongated fibroblastoid morphology, typical of BMSCs, whereas Y102 and Y202 were flattened and spread. When plated at clonal density, Y101/Y201 cells consistently formed dispersed colonies with low cell contact, characteristic of a migratory phenotype, whereas Y102 and Y202 cells formed regular, high-density, compact colonies (Figures 1B and S1D). Morphological differences were quantified by image analysis, which demonstrated that Y202 cells had a statistically significantly greater area and perimeter compared to both the Y101 and Y201 cells. Y102 cells were also larger than the Y101/Y201; the mean cell perimeters for Y102, Y202, Y101, and Y201 were 273 ± 23, 317 ± 107, 202 ± 19, and 190 ± 34 μm, and mean cell areas were 1,537 ± 132, 1,649 ± 614, 1,067 ± 92, and 857 ± 214 $\mu m^2$, respectively (Figures S1E and S1F).

We identified variations in potency following OAC differentiation assays (Figures 1C–1E). Adipogenic media for up to 21 days induced the most potent observable and statistically significant adipogenic differentiation in Y201 cells at each time point, measured using the oil red O lipid-staining assay (Figure 1C). Y101 was also observed to differentiate into adipocytes, reaching significance at day 21, but Y102 and Y202 showed severely limited, although not completely absent, adipogenic potential, which was significantly different to Y201 at day 21. In addition, there were statistically significant increases in the levels of expression of the adipogenic genes peroxisome proliferator-activated receptor (PPAR)-γ and lipoprotein lipase (LPL) in the Y201 cells cultured in adipogenic media at day 14 and day 21 compared to the time-matched untreated controls. At day 21, the expression level of both these genes was significantly lower in the three other cell lines (Figure 1C). Y101 and Y201 differentiated convincingly along osteogenic lineages and showed elevated alkaline phosphatase (ALP) activity with significantly increased calcium deposition by alizarin red staining (Figure 1D), which was statistically higher than Y102/Y202 clones, which showed minimal mineral deposition, although Y102 displayed elevated ALP activity (Figure 1D). Increases in ALP activity observed under basal conditions for Y101 and Y201 were replicated in qPCR analyses of the ALP gene (Figure S1G); further increases in ALP gene expression were clearly evident in the Y101 cell line under osteogenic conditions, while the levels in the other lines only showed modest increases. Furthermore, histological staining for elevated ALP activity was only observable in the Y101/Y201 cells (Figure S1G). Gene expression analyses for *RUNX2* showed that only the Y101/Y201 cell lines upregulated this early osteogenic marker at day 7 (Figure 1D). In response to chondrogenic induction using transforming growth factor β (TGF-β), only lines Y101 and Y201 displayed an early cell-condensation phenotype associated with chondrogenic induction (Johnson et al., 2012), whereas no condensation was observed in similar micromass culture of lines Y102 and Y202 (Figure 1E). When cultures were stained with alcian blue and stain-associated glycosaminoglycans (GAGs) were eluted and quantified, significantly different levels were observed in all cell lines at day 9 compared to their time-matched controls under basal conditions, but the highest and statistically significant GAG levels were clearly evident in the Y101/Y201 compared to the Y102/Y202 cell lines. Similarly, only the Y101 and Y201 lines displayed marked increases in total GAG production under chondrogenic conditions in the Blyscan GAG assay (Figure 1E). Gene expression analyses