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The bone marrow stromal compartment in multiple myeloma patients retains capability for osteogenic differentiation *in vitro*: defining the stromal defect in myeloma

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Summary

Defects in bone repair contribute to multiple myeloma (MM) bone disease. It is unknown whether this reflects failure of osteogenic differentiation from mesenchymal stromal cells (MSC), inherent stromal defects or mature cell dysfunction. We quantified the number of fibroblast colony-forming units (CFU-f) and osteoblast colony-forming units (CFU-ob) in freshly isolated bone marrow (BM) from healthy individuals ($N = 10$) and MM patients ($N = 54$). CFU-f and CFU-ob were present in MM BM, at comparable frequency to normal subjects, irrespective of disease stage, and the presence of bone disease. Adherent cultures from MM BM are able to differentiate into osteoblasts, as indicated by the early upregulation of *RUNX2*, *SP7*, *AXIN2* and *DLX5*, and the production of alkaline phosphatase and calcium. Coculture with MM cells failed to prevent osteogenic differentiation of adult human MSC. On the other hand, MM cells induced cell cycle progression in resting MSC in a cell contact dependent manner. This effect was confirmed using both primary CD138+ cells and MM cell lines, and was not seen with B or T cell lines. Our data confirm the presence of osteoblast progenitors and the preservation of osteogenic function in MM, however dysregulation of cell cycle control may contribute to the loss of normal bone homeostasis that ultimately results in osteolytic bone loss.

Keywords: multiple myeloma, bone disease, osteoblasts, mesenchymal stromal cells.

Osteolytic bone disease affects around 80% of all patients with multiple myeloma (MM) and is a major cause of morbidity and mortality. Increased osteoclast numbers and activity lead to bone resorption, which, in the early stage of disease, is accompanied by increased osteoblast numbers and bone formation (Bataille *et al*, 1989, 1991; Taube *et al*, 1992). It is only in the later stages of disease when osteoclastic bone resorption and osteoblast-mediated bone formation become un-coupled that bone loss ensues. Failure of bone formation and repair to keep pace with bone resorption is supported by histo-morphometric studies on MM patient BM biopsies (Bataille *et al*, 1989; Taube *et al*, 1992) and by measurement of bone formation markers *in vivo* (Carlson *et al*, 1999; Terpos *et al*, 2006). Once this un-coupling has occurred, it may never be restored, as suggested by the failure of MM bone lesions to self-repair, even in times of disease quiescence. As the survival of patients with MM continues to improve with the widespread use of novel agents (Kumar *et al*, 2008; Turesson *et al*, 2010), the quest for therapeutic strategies to promote bone formation and repair is even more urgent.

Pathways for osteoclast activation in the MM bone marrow (BM) microenvironment (RANKL, MIP-1a, interleukin-6) are well documented (Giuliani *et al*, 2005, 2008), however, the mechanism/s responsible for the failure of osteoblast numbers and activity to keep pace with osteoclastic bone resorption remain poorly understood. A role for the Wnt pathway in this process was initially suggested by the observation that elevated levels of Wnt inhibitors are expressed by MM cells, and correlate with bone disease (Tian *et al*, 2003;

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Kaiser *et al*, 2008). *In vitro* studies employing murine osteoprogenitors have demonstrated that MM cells suppress the Wnt (canonical) pathway, and this is supported by evidence from the use of anti-Dkk1 antibody in *in vivo* models (Yaccoby *et al*, 2007; Heath *et al*, 2009). Although there is no direct evidence for MM-mediated suppression of the canonical Wnt pathway in human osteoblasts (Giuliani *et al*, 2007), a role for the non-canonical pathway has recently been suggested by the demonstration that MM cells suppress the Wnt5a co-receptor, Ror2, in pre-osteoblasts (Bolzoni *et al*, 2013).

Despite these mechanistic findings, and the clinical evidence for impaired bone formation and repair in patients with osteolytic disease, studies examining the direct effect of MM cells on osteoblast function have yielded conflicting results (Karadag *et al*, 2000; Giuliani *et al*, 2005; Xu *et al*, 2012). Furthermore, while some workers report impaired osteoblastic differentiation of MM-derived mesenchymal stromal cells (MSC) (Corre *et al*, 2007; André *et al*, 2013), others have observed that patient-derived MSC exhibit normal functions, including osteogenic capability (Arnulf *et al*, 2007; Garderet *et al*, 2007). Such differences probably relate, in part, to varying assay systems, and methods of cell isolation and culture. Thus it remains unclear if the defect in bone formation is due to a failure in differentiation from MSC, or some dysfunction in mature osteoblasts.

Osteoblasts are derived by differentiation from a minority population of undifferentiated stem cells present in BM stroma, termed MSC. MSC are capable of becoming one of several phenotypes, including chondrocytes, adipocytes, muscle cells and fibroblasts, and abnormalities in this progenitor population may underlie the development of myeloma bone disease. Because these cells represent a very small fraction of BM cells, *ex-vivo* culture and expansion is invariably required, which may alter function and cellular composition of the cultures. Likewise, studies of osteoblast differentiative potential of BM cells have also generally utilized confluent cultures of MSC expanded *in vitro*, and are therefore likely to vary in the relative proportion of cell types, depending upon culture conditions and serum batch. This makes it difficult to compare between studies.

To address these issues, and to avoid such manipulations, we set out to examine the osteogenic progenitor content of freshly isolated patient MSC that have not been culture expanded and would therefore provide a snapshot of the *in vivo* function of MM patient bone marrow. We sought evidence for the impairment of osteogenic function by MM cells using both molecular and cellular assays, and employing both MM-derived and normal donor MSC. Finally we investigated the effect of MM cells on another function of adult human MSC, cell cycling. Our results indicate that the fibroblast colony-forming units (CFU-f) and osteoblast colony forming units (CFU-ob) content of MM bone marrow is not significantly different from that of normal donors, and we find no clear evidence that MM leads to impaired osteoblastic differentiation of MSC. We also report, for the first time, that MM plasma cells induce an increase in cell cycle entry in cultured human MSC.

Material and methods

Cell lines

The human myeloma cell lines (HMCL) H929, JIM3 and U266 were purchased from American Type Culture Collection (ATCC, Teddington, Middlesex, UK). The T cell line, Sup T1 and B cell line, 697 were kindly provided by Prof A. Khwaja (UCL Cancer Institute, London, UK). The KMS12BM cell line was provided by Dr T. Otsuki (Kawasaki Medical School, Okoyama, Japan) and MM1.s by Dr S. Rosen (Northwestern University, Chicago, IL, USA). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 u/ml penicillin/streptomycin.

Patients and bone marrow donors

Surplus BM samples were obtained from patients with MM or from BM donors donating for the Anthony Nolan Bone Marrow Trust, in accordance with institutional guidelines and using protocols approved by the Central London Research Ethics Committee (REC reference 03/0136, and 07/Q0502/17). BM samples were also obtained from subjects who subsequently were found not to have a haematological diagnosis. These subjects served as age-matched controls for the myeloma patients. MM patients were classified as having bone disease on the basis of at least one lytic lesion on plain skeletal X-rays or magnetic resonance imaging scan. All patients included in the study had either had no prior treatment, or were at least 6 months from their last anti-MM therapy. Cyclin D1 expression was analysed on BM trephine sections by standard immuno-histochemical methods, using rabbit monoclonal antibody SP4 (Lab Vision Products, Freemont, CA, USA). CD138+ cells were selected from freshly obtained bone marrow using the CliniMACS system as previously described (Quinn *et al*, 2011).

Isolation and culture of human mesenchymal stem cells

Bone marrow mononuclear cells (BM MNC) were isolated by centrifugation over Ficoll-Paque gradient (Amersham Biosciences, Piscataway, NJ, USA), and set up for MSC cultures (using Mesencult, Stem Cell Technologies, Grenoble, France) as detailed previously (Rabin *et al*, 2007). Initial experiments with MM BM MNC utilized the 'run-off' fraction that had been depleted of CD138+ tumour cells using the CliniMACS system, however we observed poor growth of adherent cells obtained from the 'run-off' fraction. Thereafter we set up cultures with freshly isolated BM MNC, without removing the CD138+ tumour cells. We estimated the percentage of CD138+ cells by flow cytometry, and simply adjusted the cell

numbers to standardize the number of non-CD138+ cells plated (5×10^6 cells/25 cm^2 flask). Non-adherent cells were removed after 48 h and adherent cells cultured until they reached 80% confluence. Confluent cultures were harvested with trypsin/EDTA (1%, Gibco, registered by Life Technologies, Paisley, UK), and re-plated for osteoblast differentiation.

Osteoblast differentiation

Osteogenic differentiation of MSC was induced by culture in Mesencult supplemented with 5 μg/ml ascorbic acid, 3·5 mmol/l β-glycerol phosphate and 100 nmol/l dexamethasone. Alkaline phosphatase (ALP) activity of osteoblasts was detected by a SIGMAFAST *p*-Nitrophenyl phophaste enzyme immuno-assay (Sigma-Aldrich, Gillingham, UK). Cells were also stained for ALP using Napthol AS-MX phosphate (Sigma-Aldrich) and the proportion (percentage of surface area) of ALP-positive cells was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). To assess calcium deposition, cultures were stained with alizarin red (Sigma Aldrich) after 2 weeks.

Colony forming unit (CFU) assays

Bone marrow mononuclear cells were assessed for CD138+ and CD3+ content prior to plating. Cell numbers were adjusted for the level of CD138+ infiltration, in order to seed the same number of non-MM cells for colony cultures. BM samples that comprised >20% CD3+ cells were considered to be diluted with peripheral blood, and were not used. BM MNC were seeded at 2×10^5 cells/ml in Mesencult and cultured, in duplicate (2×10^6 cells per 10 cm dish), for 14 d at 37°C, 5% CO_2 for CFU-f quantitation. For CFU-ob cultures, cells were seeded and cultured as for CFU-f, with the addition of ascorbic acid, β-glycerol phosphate and dexamethasone. At day 14 CFU-fs were stained with Giemsa following fixation with cold methanol; CFU-obs were stained with Napthol AS-MX phosphate (Sigma-Aldrich) following fixation with acetone. Colonies of greater than 50 cells were counted under direct light microscopy, and expressed as number of colonies per 10^6 MNC.

MM cells: MSC co-cultures

Mesenchymal stromal cells were cultured until 80% confluent, at which point HMCL (KMS12BM, MM1.s, H929) cells or freshly isolated CD138+ MM plasma cells were added either directly to the cultures or to a transwell configuration (Costar, 0·4 μm, 6-well plates) at a concentration of 1×10^6 cells/ml, with change of medium every 3 d.

Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated from cells using a Qiagen RNA extraction kit (Qiagen, Manchester, UK). cDNA was reverse transcribed from 500 ng of total RNA using Superscript II reverse transcriptase (Life Technologies). Gene expression was measured on an Eppendorf thermal cycler using a Power Sybr Green Mastermix (Applied Biosystems, registered by Life Technologies) reaction according to manufacturer Instructions. Primers for gene expression analysis are shown in Table S1. The $\Delta\Delta C_t$ method was used for comparative analysis of gene expression. C_t values were collected during log phase of PCR cycling. The C_t value for each gene of interest was determined for each sample and normalized to its respective C_t value of the *B2M* housekeeping gene.

Cell cycle analysis

Mesenchymal stromal cells were fixed in 2% paraformaldehyde (Sigma-Aldrich) and treated with 0·05% Triton X (Sigma-Aldrich, 2 min on ice) before staining with fluorescein isothiocyanate (FITC)-conjugated anti-Ki67 antibody (Milteny Biotech, Bisley, UK) and propidium iodide (PI; for DNA content) prior to flow cytometric analysis for DNA profiles (Cyan ADP; Dako, Ely, UK).

Western blot analysis

Cells were incubated in lysis buffer [50 mmol/l Tris-HCl, pH7·4, 150 mmol/l, 1% nonidet P-40, 05% sodium deoxycholate, 0·1% sodium dodecyl sulphate, 1 mmol/l ethylenediaminetetraacetic acid], complete protease inhibitors (Roche, Welwyn Garden City, UK) and phosphatase inhibitor cocktail (Calbiochem, Darmstadt, Germany) for 15 min on ice. 20 μg of lysate was separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Anti-phospho-pRb ($Ser^{807/811}$) was purchased from New England Biolabs (Hitchin, UK).

Statistical analysis

Data were compared using the Mann–Whitney *U*-test, and Student's *t*-test, where appropriate, using GraphPad Prism software (La Jolla, CA, USA). A *P* value of <0·05 was considered significant.

Results

MSC/Osteoblast progenitors are present in MM bone marrow at comparable frequency to age-matched subjects

The MSC/osteoblast progenitor population in MM BM was analysed by assaying for the colony-forming potential of freshly isolated BM MNC from patients and age-matched control individuals. Subject characteristics are shown in Table S2.

CFU-f, representing the stromal progenitor population in the BM and ALP positive colonies and CFU-ob, representing the osteoblast progenitor potential of this population, were counted 14 d after plating (Fig 1A). Cells in the CFU-ob

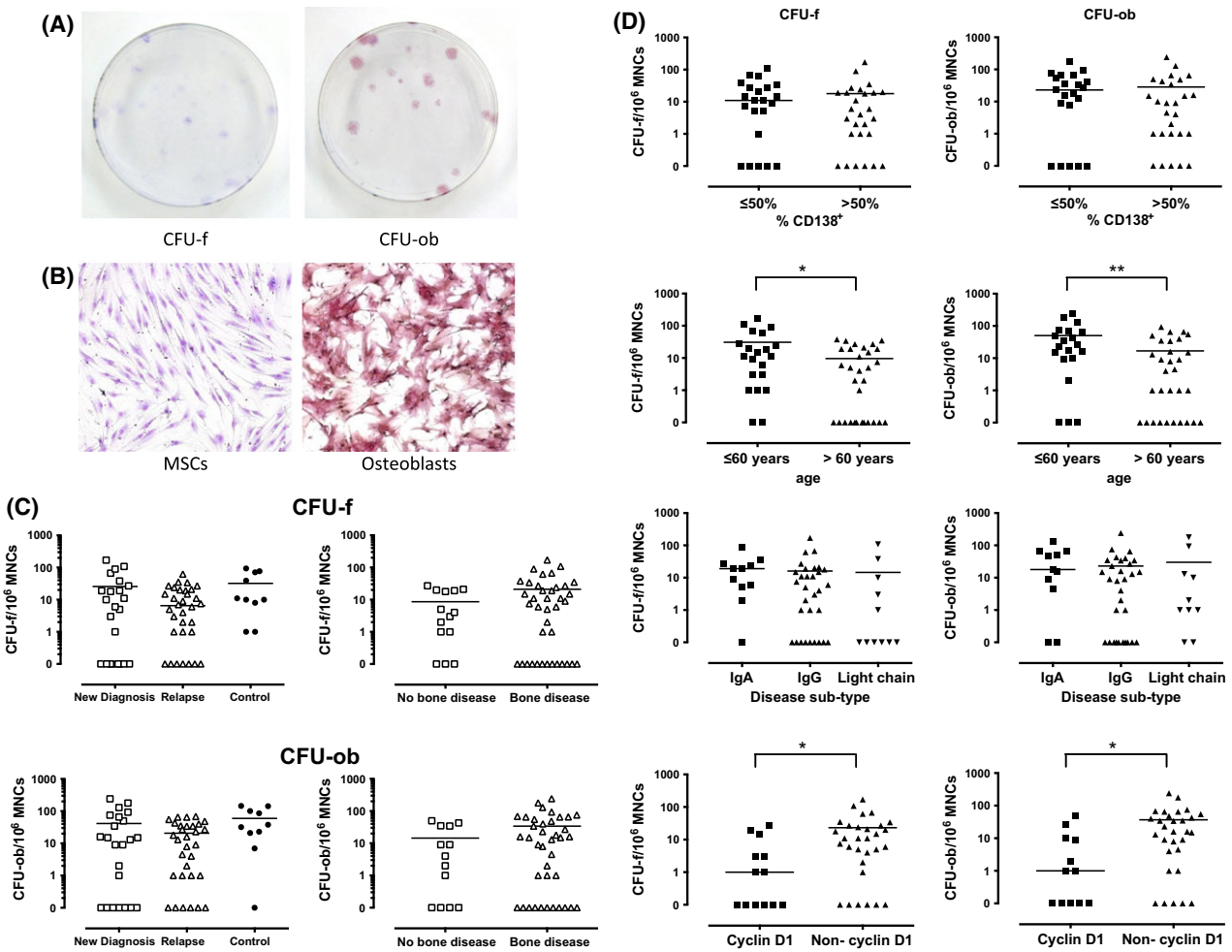


Fig 1. Comparison of CFU-f and CFU-ob frequency in MM patients and healthy age matched donors. Freshly isolated BM MNC were plated in the presence or absence of osteoblast differentiation media, and assessed for colony growth 14 d later. (A) Cultures were stained for CFU-f with Giemsa (blue) or for CFU-ob with Napthol AS-MX phosphate (red-brown). (B) Blue staining MSC are typically spindle shaped, while ALP^+ osteoblasts have a flattened cuboidal morphology. (C) Left, CFU-f and CFU-ob frequency in normal donors (control, 10) and MM patients [new diagnosis (n = 23) and relapsed disease (n = 32)]. Right, CFU-f and CFU-ob numbers in patients with osteolytic lesions (n = 39) were compared with those who had no clinical or radiological evidence of bone disease (n = 14). (D) CFU-f (left) and CFU-ob (right) frequency in MM patients according to % disease in bone marrow (%CD138+), age, isotype of disease and expression of cyclin D1. The impact of MM cell infiltration was assessed by comparing samples with low (≤50% CD138+ cells, n = 22) *versus* high (>50%, n = 27) disease burden. Disease isotypes were as follows: IgA, n = 11; IgG, n = 31; Light chain only, n = 11. Cyclin D protein expression was assessed on BM trephine sections by immuno-histochemistry (IHC), and patients with cyclin D1-positive disease on IHC (n = 13) were compared with those whose BM myeloma cells were negative for cyclin D1 (n = 30). MM, multiple myeloma; BM MNC, bone marrow mononuclear cells; MSC, mesenchymal stromal cells; CFU-f, fibroblast colony-forming units; CFU-ob, osteoblast colony-forming units; ALP, alkaline phosphatase.

colonies had typical flattened, cuboidal, ALP-positive osteoblast morphology, in contrast to the undifferentiated spindle shaped cells that comprised the ALP-negative MSC in the CFU-f (Fig 1B). Comparison of CFU-f and CFU-ob numbers between newly diagnosed MM, relapsed MM and age-matched control samples revealed no significant difference between these groups ($P > 0{\cdot}05$). Notably, CFU numbers did not differ significantly between patients with clinical bone disease and those without (Fig 1C). Interestingly, neither the percentage of tumour cell infiltration nor immunoglobulin/light chain subtype influenced colony frequency (Fig 1D). Subject age, however, had a significant impact on progenitor numbers, such that BM MNC from younger (≤60 years) subjects produced more CFU-f and CFU-ob. Thus, CFU-f frequency in subjects >60 years (10 ± 12 per 10^6 MNC, mean ± SD) was significantly lower than that in subjects ≤60 years (31 ± 45, $P = 0{\cdot}04$). Similarly, CFU-ob frequency was also lower in older subjects (16 ± 25 per 10^6 MNC vs. 50 ± 64 in younger subjects, $P = 0{\cdot}01$). Notably, BM from patients with cyclin D1-positive MM (n = 13) produced fewer CFU-f (5 ± 9 per 10^6 MNC compared with 23 ± 37 in non-cyclin D1-expressing MM, n = 30, $P = 0{\cdot}02$, Fig 1D).

CFU-ob frequency was also lower in cyclin D1-expressing patients (8 ± 15 compared with 36 ± 53 in non-cyclin D1 patients, $P = 0{\cdot}02$).

MSC from MM BM are able to undergo osteoblastic differentiation

We analysed changes in gene expression of MSC undergoing osteogenic differentiation. Normal donor MSC [at Passage 1 (P1)] and 70% confluence] were cultured in ascorbic acid, β-glycerol phosphate and dexamethasone, and expression of key genes associated with osteoblast differentiation was analysed at 24 h, 7 d and 14 d (Figs 2A and S1). We observed that *DLX5, RUNX2, SP7* (*Osterix*) and *AXIN2* were upregulated by 24 h ($P < 0{\cdot}05$ for *DLX5* and *SP7*, $P < 0{\cdot}01$ for *AXIN2* compared to control undifferentiated cells). The increase in *RUNX* levels reached significance at 7 d, remaining elevated up to 14 d ($P < 0{\cdot}01$, $P < 0{\cdot}05$ compared with control, Fig 2A). These genes are associated with commitment to the osteogenic lineage and serve as early markers of differentiation (Komori, 2006; Zhu *et al*, 2012). By 2 weeks of differentiation the expression levels of these genes had fallen (Fig 2A), while the levels of genes associated with either late osteogenic differentiation or the modulation of osteoblast function and the onset of mineralization increased [*PTH1R, BGLAP* (*Osteocalcin*) and *ALPL*, Fig S1].

Next we analysed the expression of these 'early' differentiation genes in MM patient-derived MSC after 24 h of osteogenic differentiation. MM patient MSC ($N = 6$) at P1 were grown to 70% confluence and differentiated for 24 h. Expression of each of these genes was elevated compared with undifferentiated MSC from the same patients ($P < 0{\cdot}01$ for *DLX5*, *SP7* and *RUNX2*, $P < 0{\cdot}001$ for *AXIN2*) (Fig 2B). No significant differences were observed in gene expression between differentiated patient MSC and differentiated donor MSC ($P > 0{\cdot}05$). Osteoblasts derived from MM MSC demonstrated calcium deposition as shown by positive staining for alizarin red (Figs 2C and S2), confirming their functional differentiation.

MM cells do not affect the ability of primary human MSC to differentiate ex vivo

Our data thus far indicate that MSC/osteoblast progenitors are not reduced in patient BM samples and, furthermore, that these cells are capable of differentiating into osteoblasts under *in vitro* osteogenic conditions. We next investigated if osteogenic ability of MSC is impaired in the presence of MM cells. We used conditioned media from human myeloma cell lines (HMCL), KMS12BM, H929 and MM1.s as well as the same cells in contact co-cultures with donor MSC, under osteogenic culture conditions. We did not observe any impairment in osteoblastic differentiation, as assessed by ALP enzyme immuno-staining after 7 d (Fig 3A), or by alizarin red staining after 14 d (Fig 3B). These three HMCL represent the most common *IGH* translocations found in MM patients [KMS12BM, t(11;14) NCI-H929, t(4;14) and MM1.s, t(14;16)] but similar results have been obtained with a wider panel of HMCL, using both normal donor, and MM patient-derived MSC (Fig S3).

We confirmed these findings using primary CD138+ MM cells. Primary donor MSC (P1, $N = 4$) were co-cultured with HMCL KMS12BM or MM1.s, or with freshly isolated CD138+ plasma cells from patient BM samples ($N = 5$), under osteogenic conditions, and ALP activity assessed at 7 d. No significant differences were observed between osteogenic cultures with or without MM cells (Fig 3C). ALP activity was estimated by quantifying the ALP-stained cultures using image analysis; Fig 3D shows that the presence of primary CD138+ MM cells does not affect the induction of ALP activity in osteogenic cultures. To confirm the ability of MSC to differentiate in the presence of MM cells, we utilized transwell co-cultures to analyse early differentiation genes at 24 h. Normal donor MSC were incubated in osteogenic medium, in the presence of HMCL or primary CD138+ MM cells. We observed no significant effect of HMCL or primary CD138+ MM cells on the expression of osteogenic differentiation genes in donor MSC (Fig 3E, F). We conclude that *ex vivo,* MM tumour cells do not appear to impair the osteogenic differentiation programme of human adult MSC.

Reduced CFU-f/CFU-ob ratio in MM patients

We observed a relative reduction in CFU-f numbers in MM patients. CFU-ob numbers are generally equivalent to CFU-f numbers in healthy individuals (Fig 4A). In MM patient samples, however we observed that CFU-ob numbers are consistently higher compared to CFU-f numbers in paired patient samples (CFU-f: 19 ± 30, mean ± standard deviation (SD), CFU-ob: 33 ± 44, $n = 54$, $P = 0{\cdot}04$, Fig 4B). Moreover, we observed that the ability of freshly plated MM BM MNC to form confluent cultures was reduced compared to normal donors. While all plated normal donor BM MNC formed confluent cultures, 25% of patient MM cultures failed to grow *ex vivo*.

Although CFU-ob numbers are preserved in MM BM, we observed a difference in morphology between MM BM-derived CFU-ob cultures, and those derived from normal subjects. The former contained ALP-positive spindle shaped cells (Fig 4C, left), whereas in normal donors, ALP-positive colonies consisted exclusively of cuboidal osteoblast-like cells (see Fig 1B). The spindle shaped cells in MM patient-derived CFU-ob colonies are reminiscent of colonies observed in MSC cultured with basic fibroblast growth factor (Fig 4C, right), a mitogenic factor for MSC (Sotiropoulou *et al*, 2006).

MM plasma cells induce cell cycle entry in human MSC

This led us to investigate the direct impact of MM cells on the cell cycle behaviour of MSC. Primary MSC were co-cultured

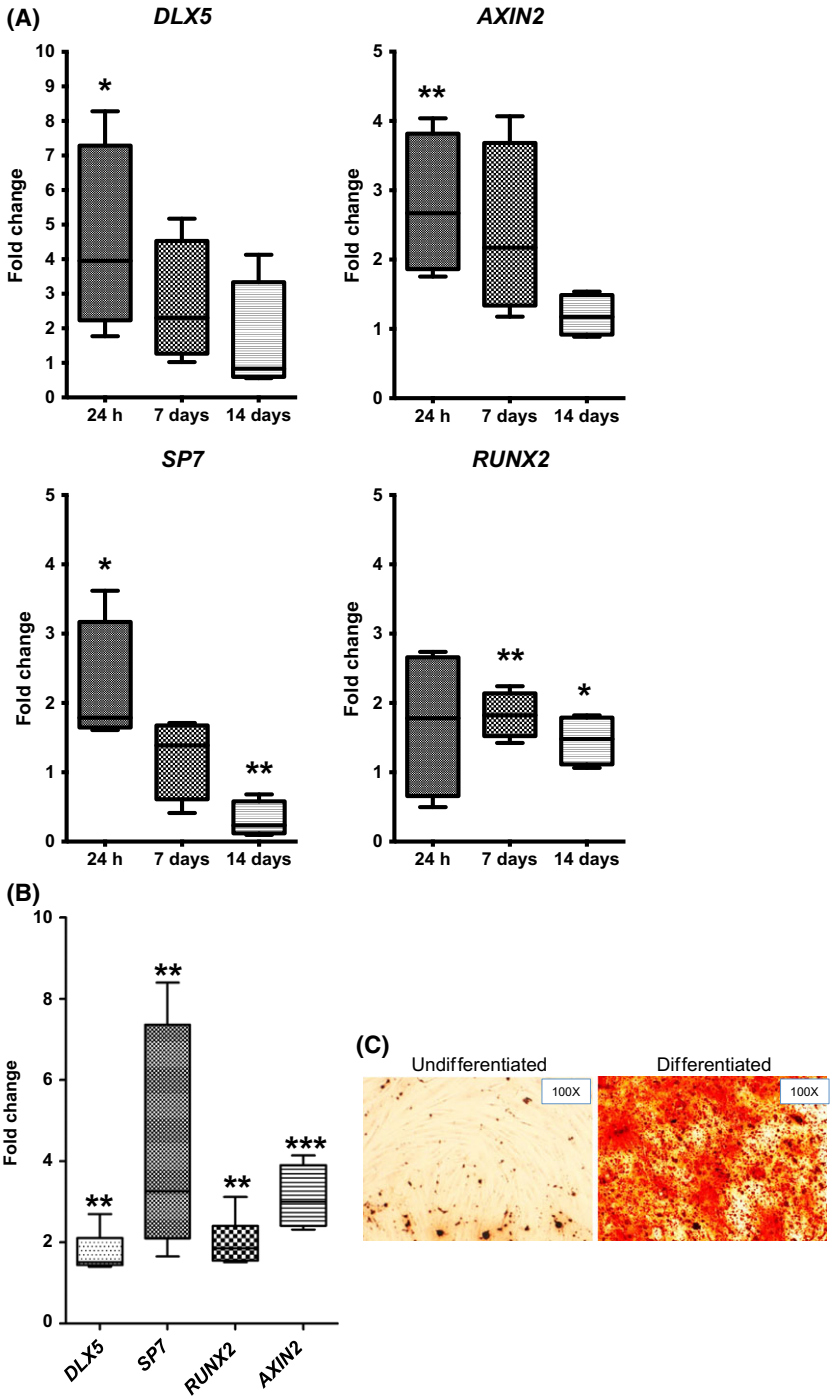


Fig 2. Osteogenic differentiation of MM MSC. (A) Donor MSC ($n = 4$) at Passage 1 were cultured in osteoblast differentiation medium, and assessed for candidate differentiation genes by real time polymerase chain reaction at 24 h, 7 d and 14 d. Effect of osteoblast differentiation on expression of *DLX5, AXIN2, SP7* and *RUNX2*. mRNA levels (Box and whisker plots) were normalized to the *B2M* house-keeping gene, and indicate the relative fold change in mRNA levels compared to control undifferentiated MSC. (B) Upregulation of these 'early' differentiation genes is shown for MSC cultures derived from BM samples taken from six different MM patients. (C) Representative osteogenic culture of MM patient MSC, stained after 14 d of differentiation (right) with alizarin red for calcium deposition. Left, undifferentiated control MSC from same patient. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$ compared to control undifferentiated cells. MM, multiple myeloma; BM, bone marrow; MSC, mesenchymal stromal cells.

with HMCL, as well as the non-MM cell lines, Sup T1 (T cell) and 697 (B cell) and cell cycle profiles analysed for Ki67 expression with PI staining for DNA content. MM cells were excluded using CD138 staining. Co-culture with HMCL cells produced a marked increase in MSC in S/G2/M phase of the cell cycle (Fig 5A, B), which was not seen with the non-MM cell lines. The presence of KMS12BM cells increased the fraction of MSC in S + G2/M from 3·9 ± 1·1% to 24·6 ± 2·8% ($P < 0{\cdot}01$, $n = 4$). Similar results were obtained for KMS12PE and MM1.S cells ($P < 0{\cdot}01$, $n = 4$ and $P < 0{\cdot}05$, $n = 5$ respectively) and other HMCL (H929, JIM3, U266, Fig S4A). Figure 5C shows cumulative data from four experiments with different MSC donors. Similar effects were observed using primary CD138+ myeloma cells. Cell cycle data from one representative experiment with five different patient-derived CD138+ cells are shown in Fig 5D, while Fig 5E summarizes the data from these five patients and four different MSC donors. Co-culture with CD138+ primary MM cells

significantly increased the proportion of MSC in S + G2/M phase of the cell cycle ($P < 0{\cdot}05$, or $<0{\cdot}01$ for each patient tested). Immunoblotting with a $Ser^{807/811}$ phospho-specific retinoblastoma protein (pRb) antibody, which recognizes CDK4/6 phosphorylated residues on pRb, confirmed increased levels of phosphorylated pRb in MSC cultured with MM cells (Fig 5F). This suggests that co-culture with MM cells induced cell cycle progression at the G1/S border. The cell cycle promoting effect of MM cells appears to require cell-cell contact, as little change in cell cycle profiles was seen when MM cells were separated from the MSC using a Transwell system (0·4 μm pore size, Fig S4B).

Discussion

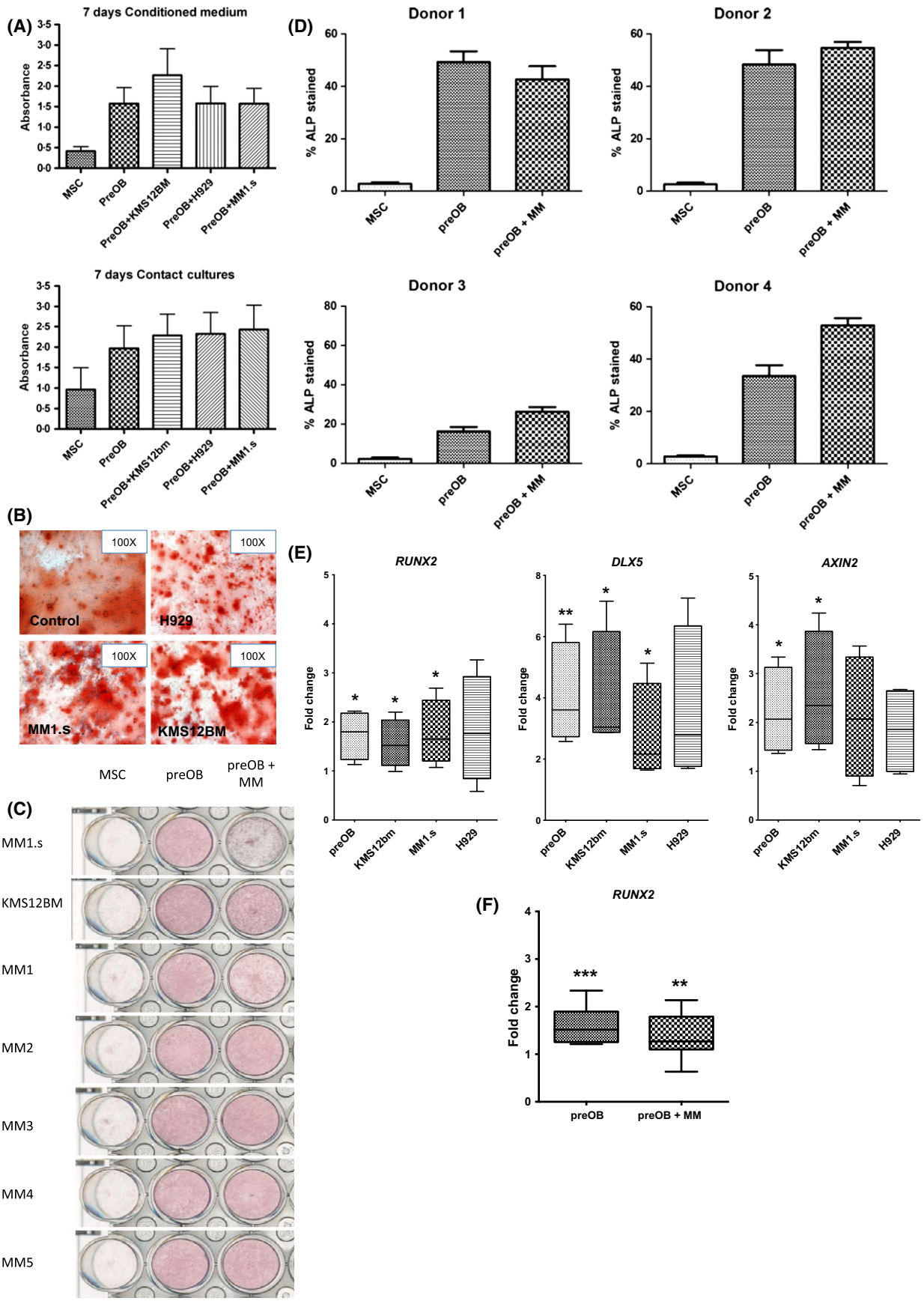
The mechanisms responsible for the unbalanced bone loss in myeloma remain incompletely understood, in particular, whether the defect in bone formation and repair is due to lack of, or dysfunctional stromal progenitors, failure to respond to osteogenic signals or suppression of mature differentiated osteoblasts or some combination thereof. We investigated these possible mechanisms by systematically analysing BM stromal progenitors and their osteogenic ability, comparing MM patients with non-diseased individuals. Here we show that MSC and osteoblast progenitor potential and frequency in freshly isolated MM BM do not differ from age-matched controls. Furthermore, MSC from MM patients are capable of osteoblastic differentiation under *in vitro* conditions, and MM plasma cells do not directly impact on the osteoblastic differentiation programme of adult human MSC. On the other hand, MM plasma cells induce cell cycle entry in MSC *in vitro*.

We observed that MM MSC are competent to execute an osteoblast programme, as evidenced by the upregulation of genes that signify an early commitment to osteoblast differentiation, as well as the induction of ALP expression and calcium deposition. These findings are consistent with some, but not all, previous reports on the subject. Corre *et al* (2007) observed that MM MSC exhibited a normal phenotype and osteoblast differentiation when assessed by ALP staining but a reduction in matrix mineralization. Arnulf *et al* (2007) reported that MM-MSC exhibited normal phenotype, differentiation capacity (osteoblast and adipocytic) and long term haematopoietic support while Garderet *et al* (2007) noted normal differentiation of MM MSC, as analysed using ALP staining and Von Kossa. In contrast, Xu *et al* (2012) observed a reduction in ALP production at 72 h during osteogenic differentiation of MM MSC, while André *et al* (2013) observed a different time course of ALP induction during osteoblastic differentiation of MM MSC. Such differences probably arise from variations in experimental conditions however, taken together, the available data suggest it is unlikely that MM MSC have a defect in osteogenesis.

The bone marrow provides a complex microenvironment in which cross-talk between different cell types regulates bone homeostasis and remodelling. It is possible that the presence of MM plasma cells disrupts this system by acting directly on resident bone marrow MSC to prevent the initiation of an osteogenic programme. We investigated this hypothesis using adult human MSC obtained from normal donors and a variety of assay systems involving both HMCL and primary CD138+ MM cells. We failed to demonstrate any direct effect of MM cells on the osteogenic programme of culture-expanded normal MSC, either in terms of gene expression changes or functional expression of ALP or calcium deposition. Our demonstration that mRNA levels of the key transcription factor, RUNX2, were not altered is in keeping with previous work from the Giuliani *et al* (2005), however, these workers reported a functional suppression of RUNX. We used HMCL from a range of different genetic backgrounds, primary MM cells from several patients and MSC from several donors, including MM patients. Under all these conditions, osteogenic differentiation was not altered by the presence of MM cells. We conclude therefore MM does not directly suppress the osteogenic programme of BM stromal progenitors.

Thus, we investigated if there was an intrinsic defect in stromal progenitors from MM patients. Several studies have reported on this subject, although the majority used

Fig 3. Effect of MM cells on the osteogenic differentiation of primary MSCS *ex vivo*. (A) Primary donor MSC were cultured under osteogenic conditions, in the presence of either HMCL conditioned media or in contact with HMCL as indicated. ALP production at 7 d was quantified by enzymatic assay, and data are mean ± sd absorbance for four donors. Control undifferentiated MSC (MSC) or differentiated cultures in absence of MM cells (PreOB) served as controls. (B) Calcium deposition, an end-point of osteoblast differentiation, as assessed by alizarin red staining, in osteogenic cultures carried out in the absence (control) or presence of HMCL (as indicated). (C) Primary donor MSC were differentiated in the presence (preOB+MM) or absence (PreOB) of MM cells for 7 d in osteogenic medium, and stained for ALP. Control cultures were undifferentiated MSC (MSC). ALP+ stained cultures of a representative MSC donor, incubated with two HMCL (MM1.s, KMS12BM), and CD138+ plasma cells from five different patients (MM1–5). (D) To quantify ALP activity, differentiated cultures from four different MSC donors were scanned, and the percentage ALP + area is given for each donor with (preOB + MM) or without (preOB) co-culture with primary CD138+ plasma cells from five patients. ALP-stained areas in control undifferentiated MSC cultures (MSC) are also given for each donor. Data are mean ± SD of % ALP-stained area. (E, F) Effect of MM cells on early differentiation genes in primary MSC under osteogenic conditions. Primary donor MSC were co-cultured under osteogenic conditions, either alone (preOB), or with (E) HMCL (KMS12BM, MM1.s, H929) or (F) primary CD138+ MM cells (pre-OB + MM). Expression levels of differentiation genes were quantified by qPCR. Data indicate the relative fold change in mRNA levels normalized to the *B2M* gene and control undifferentiated MSC. Box and whisker plot, n = 4 different MSC donors. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ compared to control undifferentiated MSC. MM, multiple myeloma; BM, bone marrow; MSC, mesenchymal stromal cells; HMCL, human myeloma cell line; ALP, alkaline phosphatase; SD, standard deviation; OB, osteoblast



culture-expanded cells. For example, Corre *et al* (2007) identified 145 genes that showed differential expression between patient and healthy control MSC, 46% of which were involved in tumour microenvironment cross-talk. Todoerti *et al* (2010) identified 45 genes differentially expressed between osteolytic and non-osteolytic patient MSC, but not osteoblast samples. André *et al* (2013) observed senescent behaviour in MM-derived MSC, and an altered genetic profile, although there was little correlation of differentially expressed genes with other reports. Such observations support the notion that functional alterations lie not within the osteoblast compartment, but in an earlier progenitor population. We wanted to obtain a 'snapshot' of the MSC compartment in un-manipulated BM by using the CFU-f assay that quantifies MSC progenitor content within the mononuclear cell fraction. Our results in a large cohort of patients indicate not only that the frequency of MSC progenitors in MM BM is not reduced, but that their ability to differentiate into osteoblasts (CFU-ob assays) is un-impaired. Our data on CFU-f and CFU-ob frequencies in normal subjects and in MM patients are comparable to published data (Giuliani *et al*, 2005; Corre *et al*, 2007). Our findings also echo those of Corre *et al* (2007), who observed that CFU-f numbers in MM patients were not significantly different from controls. Other investigators found that MM MSC produce fewer CFU-F colonies, however these studies utilized CD138-depleted cells (Garderet *et al*, 2007; Xu *et al*, 2012). This poses difficulties when comparing patient MSC function with normal donor cells that have not been subject to magnetic separation procedures. The inclusion of CD138+ tumour cells in our cultures may also produce conditions more closely representative of the MM BM *in vivo*.

Unexpectedly we observed no significant correlation in CFU-f (or CFU-ob) frequency with bone disease. We did, however, observe a decrease in CFU numbers with age, as previously reported (Stolzing *et al*, 2008). Interestingly, we noted lower numbers of CFU-f and CFU-ob in cyclin D1-expressing patients. Patients fall into disease subgroups according to recurrent *IGH* translocations and D-type cyclin expression that are validated by different molecular profiles and clinical outcomes. The molecular subgroups characterized by cyclin D1 expression, with or without the recurrent translocation t(11;14), are reported to have a higher incidence of lytic bone lesions when compared with subgroups whose plasma cells express cyclin D2 (Bergsagel *et al*, 2005), and our CFU-f data hint at the possibility that this may be due to a lack of osteoblast-initiating MSC. We found no difference between cyclin D1- and D2-expressing MM cells with regard to a direct effect on osteoblast differentiation (Fig S3 and Table S3), but there may be indirect influences on the stromal environment that are not captured in this system.

The reduction in relative CFU-f number coupled with reduced ability to form confluent MSC cultures suggests that although stromal progenitor cells are present in MM BM, and form adherent pre-osteoblast colonies in the presence of osteogenic media, there may be functional alterations in MSC progenitors. The lower proliferative capacity of MM MSC *in vitro* has been previously reported (Garderet *et al*, 2007; André *et al*, 2013), but would appear to contrast with our striking observation that MM cells induce cell cycle progression in donor MSC cultures. This was a consistent finding with both HMCL and primary CD138+ MM cells, but not with non-MM cell lines. Using a $Ser^{807/811}$ phospho-specific pRB antibody that specifically recognizes CDK4/6 phosphorylated residues (Zarkowska & Mittnacht, 1997), we show that phosphorylated pRb levels are increased in MSC exposed to MM cells, suggesting that the increase in S/G2/M fraction is mediated primarily by increased activity of the cyclin D/CDK4/6 complexes. Increased cell cycling of MM MSC is consistent with an earlier report, which used rodent BM cells, that co-culture with HMCL increased osteoblast recruitment and CFU-ob formation (Karadag *et al*, 2000). It is also consistent with the observation that, early in the disease course, patients have increased bone formation rates (Bataille *et al*, 1989). A study of the MM niche in an immunodeficient xenogeneic model describes a re-organization of the extracellular matrix components, with a proliferation of nestin+ cells (Iriuchishima *et al*, 2012), nestin being one of the markers of the MSC progenitor compartment. Finally, Noll and colleagues recently reported an increase in MSC numbers following tumour cell inoculation in the C57BL/KaLwRij murine model of MM (Noll *et al*, 2013). Taken together, these observations are consistent with the hypothesis that the presence of MM cells initially stimulates proliferation of stromal progenitors, which may help to establish the myeloma niche. Chronic exposure to proliferative stimuli, however, could render these cells hypo-proliferative when removed from the myelomatous marrow, accounting for our and others observations (see above). Notably, among the genes differentially expressed in MSC from patients with osteolytic disease are some involved in cell cycle regulation, adhesion and migration (Todoerti *et al*, 2010).

Meanwhile the search for a mechanistic basis for the impaired bone formation in MM patients continues. While MSC and pre-osteoblasts from MM patients are competent to execute an osteogenic programme *in vitro*, their functional competence *in vivo* relies also on the ability to migrate and localize to sites of bone lysis and on the production of chemotactic factors in the local environment. An examination of such processes may shed light on the bone repair problems *in vivo*. Our work has focused on the MSC compartment and the osteoblastic potential thereof, however, a further possible mechanism for impaired bone formation is the de-regulation of mature, end-stage bone-forming cells, such as has been suggested for osteocytes (Giuliani *et al*, 2012).

In summary, we observed, in a large cohort of MM patients, that the incidence of MSC progenitors and their

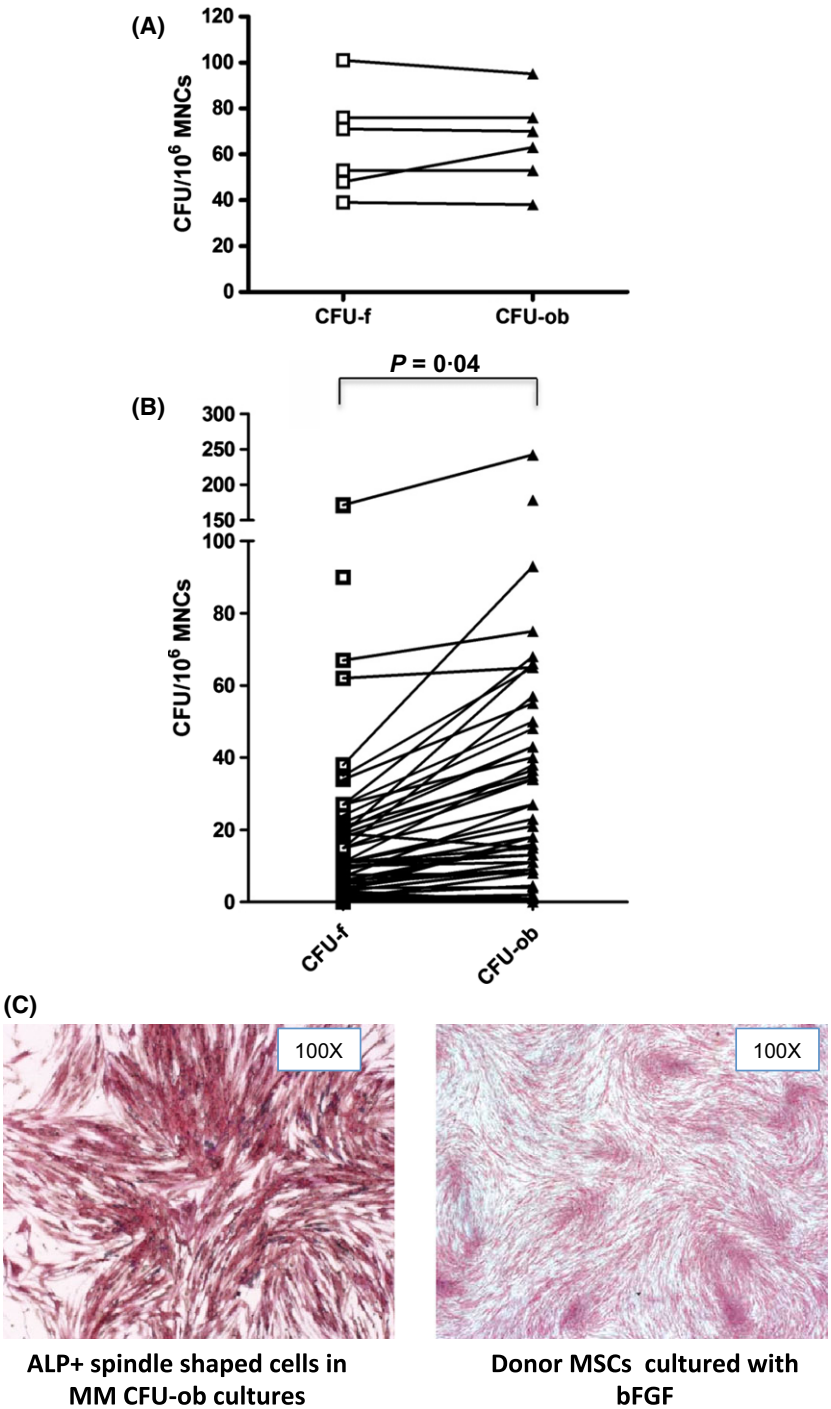


Fig 4. Functional abnormalities of MM MSC progenitors *in vitro*. Freshly isolated donor and MM patient BM MNC were plated in the presence or absence of osteoblast differentiation media, and assessed for colony growth 14 d later. It was observed that (A) healthy donors generated comparable numbers of CFU-f and CFU-ob colonies while (B) CFU-f numbers were consistently lower than CFU-ob numbers in myeloma patients, $P = 0{\cdot}04$, $n = 54$. (C) Colonies of ALP + spindle shaped cells were observed in some MM patient CFU-ob cultures (left). These colonies resemble those produced when donor MSC are cultured in the presence of basic fibroblast growth factor (bFGF, right). MM, multiple myeloma; BM MNC, bone marrow mononuclear cells; MSC, mesenchymal stromal cells; CFU-f, fibroblast colony-forming units; CFU-ob, osteoblast colony-forming units; ALP, alkaline phosphatase.

subsequent ability to differentiate into osteoblasts is unimpaired. Our observations, that the MM BM contains stromal progenitors capable of osteogenic differentiation, provides encouraging support for the clinical use of bone anabolic agents, many of which are in clinical trials (Vallet & Raje, 2011). Such agents may serve to sustain an osteogenic environment to promote local bone formation and repair in osteolytic areas. We did not find that MM cells directly impair MSC differentiation in response to *in vitro* osteogenic conditions. Our observation that MM cells stimulate cell cycling in BM MSC is consistent with reports of osteoblast recruitment and increase in MSC numbers *in vivo*. Over time, however, exposure to chronic mitogenic stimuli may lead to local defects in bone repair, perhaps due to altered adhesive and migratory function. Importantly, it should be possible to exploit the intrinsic, and preserved, osteoblastic function clinically to promote repair.

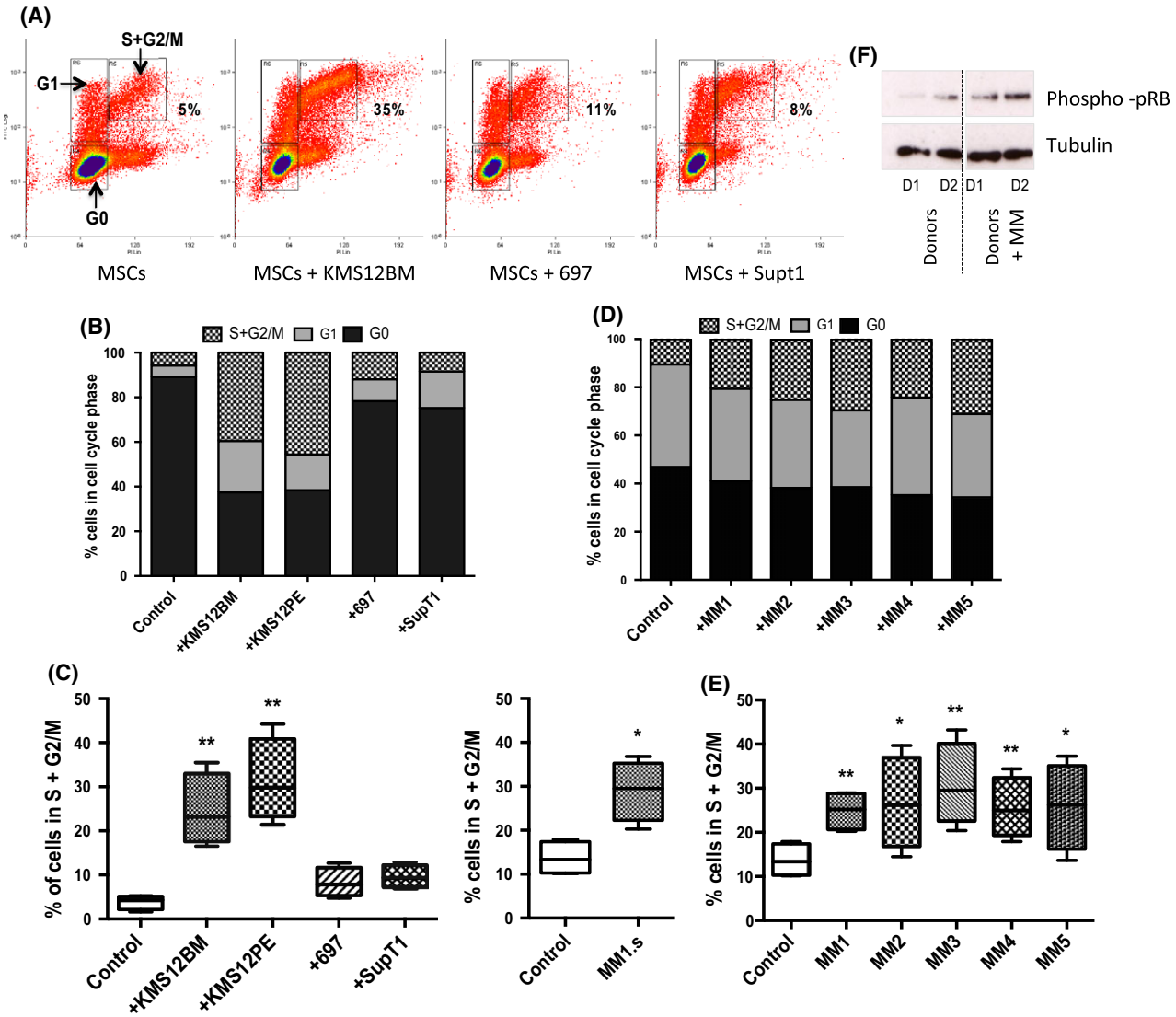


Fig 5. MM plasma cells induce cell cycle progression in adult human MSC. Serum-starved MSC ($N = 4$) were co-cultured with HMCLs: MM1.s, KMS12BM and KMS12PE, with the B-cell line, 697 or T-cell line SupT1, or with primary CD138+ MM tumour cells for 24 h. Cell cycle behaviour of MSC was assessed by Ki67/PI staining, using CD138 staining to exclude MM cells. (A) fluorescence-activated cell sorting plots from one representative experiment showing the cell cycle plots for MSC cultured alone (MSC) or with cell lines as indicated. The location of cells in different phases of the cell cycle is indicated in the left histogram, and the percentage of cells in S/G2/M is given for each condition. (B) Cell cycle data from one representative experiment. MSC were cultured alone or with each of the cell lines as indicated. (C) Summary of results from experiments with different MSC donors, four donors on left histogram with HMCL and non-MM lines, and three donors on right, with MM1.s cells; proportion of cells in S + G2/M, box and whisker plots. (D) Primary CD138$^+$ MM cells were cocultured with MSC (+MM1–5) and cell cycle data are given for one representative experiment with five different primary MM CD138+ cells. Control, control cultures without MM cells. (E) Summary of results from separate experiments with four different MSC donors and five different patient samples (MM1–5), data as for C). (F) MSCS cultured alone (donors) or co-cultured with CD138+ MM cells (donors + MM) for 24 h were assessed for phospho-pRB levels by Western blotting. Two different MSC donors (D1, D2) were used. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ compared to control MSC cultured alone. MM, multiple myeloma; BM, bone marrow; MSC, mesenchymal stromal cells; HMCL, human myeloma cell line.

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research study, KY designed the research study, analysed the data and wrote the paper.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Expression of 'late differentiation' genes during osteogenic differentiation of MSC.

Fig S2. Alizarin-stained cultures of MSC from normal donors (3) and Myeloma patients (4), showing undifferentiated, and effect of 14 days culture in osteogenic medium.

Fig S3. ALP activity (by enzyme immunoassay) in normal donor MSC (Donor 1) and 2 patient-derived MSC co-cultured with HMCL as indicated, after 7 days in osteogenic medium (preOB) or normal Mesencult (MSC).

Fig S4. Effect of myeloma cells on cell cycle behaviour in MSC.

Table S1. primer sequences used for real time PCR.

Table S2. MM patient and healthy donor characteristics.

Table S3. Myeloma cell lines : D-type cyclin/s expressed and genetic lesions.

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