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Myeloma plasma cells alter the bone marrow microenvironment by stimulating the proliferation of mesenchymal stromal cells

*Short Title: Multiple myeloma alters the bone microenvironment*

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Abstract:

Multiple myeloma is an incurable haematological cancer characterised by the clonal proliferation of malignant plasma cells within the bone marrow. Numerous studies suggest that the myeloma plasma cells occupy and alter the stromal tissue of the bone marrow as a means of enhancing their survival and growth. However, the nature and magnitude of the changes to the stromal cell tissue remain to be determined. In this study, we used mesenchymal stromal cell and osteoblast-related cell surface marker expression (STRO-1 and alkaline phosphatase, respectively) and flow cytometry to enumerate mesenchymal stromal cell and osteoblast numbers in bone marrow recovered from myeloma patients at the time of diagnosis. Using this approach, we identified an increase in the number of STRO-1 positive colony forming mesenchymal stromal cell and a concomitant decrease in alkaline phophatase osteoblasts. Notably, this increase in mesenchymal stromal cell numbers correlated closely with plasma cell burden at the time of diagnosis. Additionally, in comparison with the osteoblast population, the STRO-1+ mesenchymal stromal cell population was found to express higher levels of plasma cell- and osteoclast- activating factors, including RANKL and IL-6, providing a mechanism by which an increase in mesenchymal stromal cell may promote and aid the progression of myeloma. Importantly, these findings were faithfully replicated in the C57BL/KaLwRij murine model of myeloma, suggesting that this model may present a unique and clinically relevant system in which to identify and therapeutically modulate the bone microenvironment and in turn, alter the progression of myeloma disease.
Introduction:

Multiple myeloma (MM) is characterised by the clonal proliferation of malignant plasma cells (PC) within the bone marrow (BM). MM accounts for approximately 1% of all cancers and is the second most common haematological malignancy after non-Hodgkin’s Lymphoma. The main clinical manifestations of MM are the development of devastating osteolytic bone lesions, bone pain, hypercalcaemia, renal insufficiency, suppressed haematopoietic function, reduced polyclonal immunoglobulin production, and increased BM angiogenesis. MM encompasses a spectrum of clinical variants ranging from benign MGUS and smouldering/indolent MM, to more aggressive, disseminated forms of MM and PC leukaemia. Despite recent advances in protease inhibitor and immunomodulatory drug-based therapies, MM remains largely incurable.

While aberrant BM microenvironments have been implicated as playing an inductive role in some haematopoietic diseases (1-3), in most instances the BM provides an environment that is permissive for the proliferation of haematopoietic neoplasms. For example, B-cell tumours, including chronic lymphocytic leukaemia and lymphoma exploit the normal BM microenvironment to support their survival, proliferation and resistance to chemotherapeutic agents (4). Similarly, MM PC also modify their BM microenvironment, via the production of cytokines and growth factors and by direct cell-cell interactions, to create a milieu that supports their survival (5, 6). Furthermore, in response to MM PC the tumour-associated mesenchyme produces numerous pro-osteoclastogenic cytokines that increase osteoclast (OC) recruitment and OC-mediated bone loss at sites proximal to the PC tumour (5, 7, 8).

Previous studies have shown that mesenchymal stromal cells (MSC) and osteoblasts (OB) isolated from MM patients are phenotypically and functionally altered compared with those recovered from healthy, age-matched donors (9-12). In vitro culture studies show that the osteogenic capacity of MM patient-derived MSC is impaired, when compared with that of normal MSC (13). In addition, several recent microarray studies have shown that MSC from MM patients display unique gene expression signatures compared with those recovered from normal donors,
including an up-regulation of amphiregulin, IL-1β and IL-6 expression; factors that may increase the proliferation of MM PC (13-15). Notably, these genetic differences were not found in MM patient-derived OB (15), indicating that MSC may represent a key stromal cell population with the capacity to influence the growth of malignant MM PC. This has led investigators to examine whether MM patients show evidence of elevated MSC numbers following MM PC infiltration into the BM. To this end, conflicting reports suggest that, relative to healthy donors, MSC numbers are unchanged (13), reduced (14) or increased (16) in MM patients.

In an attempt to address these contradictory findings, we utilised magnetic activated cell sorting and flow cytometry to prospectively isolate and enumerate MSC in BM recovered from diagnosis MGUS and MM patients and healthy, age-matched controls. Notably, we observed an increase in MSC numbers in both MGUS and MM patients compared to controls, and this increase in MSC numbers was closely correlated with PC burden at the time of diagnosis. In addition, using the 5TGM1/C57BL/KaLwRij mouse model of myeloma, previously shown to closely mimic human disease (17-20), we observed an increase in MSC numbers, and a concomitant decrease in OB numbers, which correlated closely with intramedullary tumour burden. This model therefore provides a unique opportunity to investigate the progression of MM disease in an in vivo setting and to evaluate novel therapeutics designed to target the BM microenvironment.

**Methods:**

**Human Bone Marrow Samples**

Aspirates of bone marrow (BM) were obtained from MM, MGUS and normal donors in accordance with Institutional Ethics Committee approved guidelines (Royal Adelaide Hospital Ethics # RAH REC study #010516 and REC study #030206). Bone marrow mononuclear cells (BMMNC) were prepared from BM aspirates by density gradient separation, as described previously (21).

**Colony Forming Unit-Fibroblast (CFU-F) Assay**
CFU-F assays were performed at plating densities ranging from 0.1 to 1 x 10^4 unfractionated or immune-selected BMMNC per cm^2 in triplicate 6-well plates over a 12 day period, as previously described (see supplementary methods) (21).

Animals

C57BL/KaLwRij mice were bred and housed at the Institute of Medical and Veterinary Science (IMVS) Animal Care Facility. The studies were performed in accordance with IMVS-Animal Ethic Committee approved procedures. Six-week-old C57BL/KaLwRij mice received 5x10^5 luciferase/GFP 5TGM1 cells in 100 μL of sterile PBS via the tail vein. Intramedullary tumour growth was determined by weekly bioluminescent imaging. Briefly, mice were administered luciferin (150 mg/kg) i.p. and live-imaging performed using the Xenogen IVIS 100 bioluminescence imaging system (Caliper Life Sciences, Hopkinton, MA).

Flow Cytometric Analysis and Cell Sorting

Following enzymatic digestion (see supplementary methods), stromal cells were flow cytometrically sorted on the basis of STRO-1 and alkaline phosphatase expression as previously described (22) (see supplementary methods).

Compact bone-derived cells (see supplementary methods) from tumour-bearing and control mice were resuspended at 2 x 10^7 cells/mL in 2% FCS/2 mM EDTA/PBS solution and stored on ice throughout processing. Cells were blocked with mouse gamma globulin at 1:100 for 30 minutes. Cells were stained for 30 minutes with an antibody cocktail comprised of biotin labelled rat anti-mouse antibodies: B220, CD3, CD4, CD5, CD8, Gr1 and Ter119 (BioLegend, San Diego, CA) and CD11b (eBioscience, San Diego, CA), rat anti-mouse APC eFluor780 CD45 (eBioscience), rat anti-mouse PerCP/Cy5.5 CD31 (Biolegend), rat anti-mouse PE-Cy7 Sca-1 (Becton Dickinson) and rat anti-mouse PE-CD51 (Biolegend). Cells were washed twice and incubated with streptavidin-APC (Life Technologies, Victoria, Australia) for 30 minutes, washed and resuspended at 2 x 10^7 cells/mL. Fluorogold was added prior to sorting to facilitate live:dead gating. Cells were sorted on a
Becton Dickinson Aria. Cell sub-populations were defined as follows: Lin-CD45-CD31-Sca-1-CD51- MSC (23-25) and Lin-CD45-CD31-Sca-1-CD51+ OB (26, 27).

**RNA Isolation and real-time PCR**

Total cellular RNA was isolated from cell populations using standard procedures. cDNA was generated and real-time PCR performed using specific primers for IL-6, RANKL and OPG (see supplementary methods).

**Results:**

The incidence of STRO-1+ mesenchymal stromal cells (MSC) is higher in MM and MGUS patients and is correlated with more severe disease

Bone marrow mononuclear cells (BMMNC) were recovered from the bone marrow of healthy donors (n=9) and MGUS (n=7) and MM (n=17) patients at diagnosis using ficoll-density gradient separation. The number of colony forming unit fibroblast (CFU-F), a measure of MSC number (21), was evaluated in single cell suspensions of total BMMNC or BMMNC cells enriched for CFU-F using the MSC-specific monoclonal antibody, STRO-1 and magnetic activated cell sorting (MACS). STRO-1 is a well characterised MSC antigen which has been used extensively to enrich for a population of immature, multi-potent MSC (28, 29). The pre-enriched and STRO-1 selected BMMNC were plated at low density and the number of CFU-F determined as described in Materials and Methods. As seen in Figure 1, while there was a trend toward an increase in CFU-F numbers in the MGUS and MM patients relative to healthy controls, this increase was not significant. However, when STRO-1 was used to prospectively isolate MSC, there was a significant increase in the number CFU-F in BMMNC from MGUS and MM patients compared with age-matched healthy controls (86+/-23 and 152+/- 53 compared with 222+/- 27, p<0.05 and p<0.01, ANOVA, respectively).
Explant cultures of healthy donor and MM-derived stromal cells cultivated from primary 
CFU-F were cultured for 3 passages in vitro (approximately 8-10 population doublings). Dual 
colour flow cytometry using the MSC-associated antibody, STRO-1, in conjunction with an 
antibody specific for the OB marker alkaline phosphatase (ALP) was used to enumerate the MSC 
and OB content in each of the explant cultures (22, 30). As seen in Figure 2A and B, using this 
approach, explant cultures could be separated into four populations characteristic of different stages 
of OB differentiation (22), with the least differentiated MSC-like population being STRO-1⁺ALP⁻, 
and the most differentiated OB-like being STRO-1⁻ALP⁺. A significantly greater number of STRO-
1⁺ALP⁻ cells (p<0.05, ANOVA) were present in explant cultures from MM patients compared with 
healthy donors, consistent with the increased CFU-F potential seen in MM patient samples. 
Notably, this increase in the MSC population was accompanied by a concomitant decrease in the 
number of STRO-1-ALP+ osteoblasts (Figure 2C). Furthermore, as seen in Figure 3A, a positive 
correlation (R²=0.4976, p<0.01, Spearman Rank) was observed when the percentage of STRO-
1⁺ALP⁻ MSC (of the total population) was compared with the number of CD138+ PC (as a 
percentage of total BM cells) present in the BM at diagnosis. In contrast, a significant negative 
correlation (R²=0.3125, p<0.1, Spearman Rank, p<0.01) between PC number and ALP+ OB number 
was observed (Figure 3B), indicating that the BM stromal tissue is manifestly altered following 
exposure to MM PC, leading to an increase in MSC numbers and a decrease in OB numbers.

MSC are a rich source of plasma cell and osteoclast activating factors

In order to determine a mechanism through which increased MSC numbers may promote 
MM disease development and progression, we investigated the mRNA expression of a select 
number of plasma cell- and osteoclast-activating factors IL-6, RANKL and OPG, in FACS sorted 
populations of STRO-1⁺ALP and STRO-1⁻ALP+ cell populations from healthy individuals (Figure 
4A). As seen in Figure 4B, the STRO-1⁺ALP⁻ MSC population exhibited significantly higher 
expression of IL-6 and RANKL compared with the STRO-1⁻ALP+ OB population (p<0.05 and
p<0.05, respectively, t-test). In contrast, the STRO-1–ALP⁺ OBs expressed significantly higher levels of the OC-inhibitory factor OPG (p<0.001, t-test).

**Multi-colour flow cytometry identifies an increase in MSC within the C57BL/KaLwRij murine model of MM disease**

The C57BL/KaLwRij strain of mice was reported to develop myeloma in 0.5% of aging animals, a phenomenon not observed in the related C57BL/6 strain (31). A number of plasma cell lines have subsequently been derived from the bone marrow of these mice that, upon re-introduction to the C57BL/KaLwRij mice via the tail vein, result in complete penetration of disease and the development of systemic myeloma which closely mimics human disease (17, 19, 20, 32).

Six-week-old C57BL/KaLwRij mice were intravenously inoculated with 5x10⁵ luciferase/GFP-labelled 5TGM1 myeloma plasma cells via the tail vein. The use of luciferase labelled cells allowed us to monitor the progression of the disease using bioluminescent imaging techniques (33). Tumour progression was monitored at 14 and 28 days post-tumour cell inoculation by live animal bioluminescent imaging (Figure 5A). On day 28-post tumour inoculation, mice were humanely killed and their long bones (tibiae and femur) extracted for analysis of MSC numbers. The long bones were chosen as a source of MSC as the compact bone, rather than the bone marrow, has previously been shown to be the major source of MSC within the adult mouse (23-25). Compact bone-derived cells were isolated by enzymatic digestion of bone fragments, followed by depletion of mature cells of the haematopoietic lineage using a cocktail of antibodies specific to the B220, Gr-1, Mac-1, CD3, CD4, CD5, CD8 and Ter-119 lineage markers. Lineage-negative (Lin⁻) cells were subsequently analysed based on the expression of CD45, CD31, CD51 and Sca-1 phenotypic markers and viable cells were gated by fluorogold exclusion (34).

Initially, the viable cell populations were sorted and the GFP Lin CD45⁻CD31⁻ population of cells was plated at low density for subsequent determination of CFU-F. Sorting for GFP negative cells ensured the exclusion of contaminating tumour cells. The exclusion of cells expressing CD45, CD31 and haematopoietic lineage markers ensured the exclusion of contaminating macrophages,
haematopoietic and endothelial cells. The GFP Lin'CD45'CD31' cell population derived from tumour-bearing mice showed a significant increase in CFU-F at 28-days post tumour cell inoculation compared to control mice (p<0.05, t-test) (Figure 5B). Incorporation of antibodies directed toward the CD51 and Sca-1 phenotypic markers allowed for more detailed FACS analysis of cell populations present within the compact bone and the relative proportions of OB (Lin'CD45'CD31'CD51'Sca-1') and MSC (Lin'CD45'CD31'CD51'Sca-1+) were subsequently determined (Figure 6A-D). At 28-days post-tumour inoculation, tumour-bearing mice exhibited a significant increase (p<0.05, t-test) in the proportion of MSC within the bone and significant decrease (p<0.05, t-test) in OB numbers compared with controls (Figure 6E and F). Similarly to the studies detailed in Figure 4, gene expression analysis of murine MSC from control animals show a significant increase in IL-6 expression when compared with cells committed to the OB lineage (Figure 7).

**Discussion:**

Previous studies have shown that MM PC are critically dependent upon stromal cell interactions and signalling pathways within the bone microenvironment for MM disease development. This is due to direct cell-cell interactions, which mediate malignant PC growth and survival, as well as signalling through various cytokines and growth factors that support the progression of MM tumours (6). In this study we have shown, for the first time, that there are measurable changes in the stromal cell composition of the BM compartment that follows the development and progression of MM. Moreover, we provide evidence that the C57BL/KaLwRij mouse model of myeloma faithfully replicates these findings, and highlights its value as a pre-clinical model to investigate novel therapeutic strategies that may target the bone microenvironment in myeloma.

Using the monoclonal antibody STRO-1 and MACS, we identified a significant increase in the proportion of MSC within the bone marrow of MM patients, potentially at the expense of
osteogenesis (Figure 1 and 2). Previous studies have reported conflicting results regarding the presence of MSC within the bone of MM and MGUS patients compared to healthy donors. Two studies utilised a plastic adhesion method of MSC isolation and showed a decrease (14) or no change (13) in the number of MSC in MM patients compared to healthy controls. However, a study by Jones and colleagues used flow cytometry to isolate a pure population of CD45<sup>lo</sup>D7- FIB<sup>+</sup>CD271<sup>+</sup> MSC from BM aspirates from MM or MGUS patients, or normal controls, and showed that MSC numbers are 2-fold higher in BM aspirates from MGUS and MM patients, compared with age-matched controls (16). Recent reports have suggested that isolation of MSC by flow cytometry using antibodies directed toward specific cell surface antigens provides a much more robust and reliable method of identifying immature, multi-potent MSC than the historically popular method of plastic adhesion, while simultaneously avoiding contamination of cultures with macrophages and other cells of the haematopoietic lineage (28, 35). This supports the validity of our findings and suggests that the methodology employed to isolate MSC may significantly impact on results and is in fact likely to account for differences in conclusions drawn by different studies.

Our data also shows that the patients exhibiting the greatest proportion of MSC within the bone marrow also present with the greatest PC burden (Figure 3), suggesting that the MSC population is better able to support MM PC growth and myeloma disease development. A recent study by Xu et al. showed that re-introduction of <i>ex vivo</i> expanded MSC in a mouse model of myeloma resulted in increased severity of disease and decreased survival (36), which coupled with our data suggest that the presence of excess MSC may promote MM progression and perhaps be indicative of a poor prognosis.

It is not surprising perhaps, that an increase in MSC is linked with MM disease in human patients and is likely to correlate with disease severity. Over recent years there have been numerous studies that have investigated the effects of MSC on MM PC and OB alike. In this study we demonstrate an increase in MSC <i>in vivo</i> which is accompanied by a decrease in OB numbers within the BM of MM patients compared to MGUS and healthy controls (Figure 2C). This is consistent
with *in vitro* studies that have shown that malignant PC are a source of a number of inhibitors of OB-differentiation including the Wnt-pathway antagonists Dickopf-1 (DKK-1), soluble frizzled related protein (sFRP) and the OB-inhibitory cytokines, IL-7 and IL-3 (37-39). Moreover, the decrease in OB may also be due to an increase in apoptotic signalling in OB in the presence of MM PC (40, 41). This decrease in active OB-function is likely to contribute to the osteolytic bone disease commonly observed in MM patients with advanced disease.

In normal B-lymphocyte development, HSC differentiate into B-cell precursors in BM niches comprised of OB and MSC. The cells within these niches express various growth factors and adhesion molecules, including CXCL12, Flt3 ligand, IL-7, integrins, VCAM-1 and N-cadherin, which stimulate B-cell survival and proliferation (42). In keeping with this, we show high expression of factors RANKL and IL-6 from STRO-1+ MSC (Figure 4). Although differences are noted in the expression of RANKL between human (Figure 4) and mouse (Figure 7) samples, MSC have a greater RANKL:OPG expression ratio in both human and mouse when compared to osteoblasts. This is indicative of an osteoclast- and MM-supportive environment (43). The expression of all these factors, coupled with the observed increase in MSC numbers in MM patients, may further influence the BM stromal cell composition. Our findings are consistent with previous reports that indicate an increase in IL-6 from MM patient-derived MSC (9, 36, 44). RANKL expression has also been demonstrated to be increased in MM patients (5, 7) and MM stromal cells are a significant source of RANKL in MM patients (30, 43).

The 5T/C57BL/KaLwRij mouse model of myeloma presented in this study has been widely used to investigate various aspects of MM, including but not limited to, the homing and migration of MM plasma cells (20), the role of BM-derived stromal cells in mediating MM plasma cell growth *in vivo* (45), osteolysis in MM disease (46) and the identification of novel genes that exhibit altered expression in MM (47). For the first time, we show using the C57BL/KaLwRij model that the cellular composition of the bone microenvironment is manifestly changed in the presence of tumour (Figure 5 and 6). Importantly, the changes observed in this mouse model of myeloma,
specifically an increase in MSC and a decrease in OB in the presence of tumour, mirror those seen in patients (Figure 2). Coupled with evidence that this model closely mimics the human disease in other respects, specifically in relation to the osteolytic bone disease commonly associated with MM, our data supports the use of this model to identify novel pathways for therapeutic intervention. In particular, in combination with the increasing focus on the bone microenvironment and its importance in the development of disease and maintenance of malignant PC, the C57BL/KaLwRij model may provide a means of investigating the clinical benefits of targeting the bone microenvironment as a novel treatment modality for MM. It would be of interest to investigate the validity of modulating the cellular composition of the bone microenvironment through pharmacological targeting of the osteogenic pathway.

A number of small molecules have already been identified that may increase osteoblastic differentiation of MSC. BIO (6-bromoindirubin-3’-oxime) is a GSK3β inhibitor that can increase expression of early osteogenic markers in MSC (48, 49). BIO has been shown to increase bone volume in wild-type mice and may function to decrease MM tumour burden specifically in the context of the bone microenvironment (50, 51). Similarly, purmorphamine, a Hedgehog (Hh) pathway agonist that functions through the receptor Smoothened to enhance osteogenesis of murine-derived MSC (52, 53) has been shown to have a pro-osteogenic effect on human-derived MSC (54, 55). Another small molecule, decalpenic acid (CR37010), was also identified as having the capacity to induce early osteogenic markers in murine pluripotent MSC (56, 57) however this has not yet been demonstrated in a human setting. These molecules, and others, represent potential therapeutics that may be further investigated, specifically in relation to their capacity to modulate the cellular composition of the bone i.e. to decrease MSC and increase OB. In addition, a recent publication by Kaiser et al. has demonstrated that the proteasome inhibitor bortezomib, which is currently used as an effective anti-myeloma therapy (58), is able to stimulate osteoblastic differentiation of human MSC in vitro, particularly in the presence of exogenous vitamin D (59). In view of our data showing that there is an increase in the incidence of MSC in
MM patients, targeting both the malignant PC and the surrounding microenvironment may provide improved patient outcomes. This may in turn be beneficial in maintenance therapies to prevent relapse in patients that have successfully responded to first round treatment by providing an environment less conducive to the growth and development of malignant PC.

In conclusion, we have identified an increase in the proportion of MSC present within the bone of MM patients, indicating that MM does in fact alter the cellular composition of the bone. The ability to replicate these stromal cell changes in C57BL/KaLwRij mice provides evidence that this system represents a unique model for identifying novel pathways and investigating the efficacy of treatment strategies that directly target the bone microenvironment.

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Authorship and Disclosures:

JN & AZ were the principal investigators, JN, SW, CT, HW, JQ & LP performed laboratory work for this study, LBT, AE, SG, & AZ facilitated co-ordination of research and access to patient samples, KP performed and optimised flow cytometry, JN and AZ wrote the paper. The authors report no potential conflicts of interest.
References


Figure Legends:

**Figure 1.** Clonogenic CFU-F numbers are elevated in MGUS and myeloma patients. Bone marrow mononuclear cells (BM MNC) were recovered from healthy donors (n=9), MGUS patients (n=8) and myeloma patients (n=17) by Ficoll-density gradient separation. The colony forming unit-fibroblast (CFU-F) potential of total BM MNC (PRE) and magnetic activated cell sorted (MACS) STRO-1+ BM MNC (STRO-1+) was assessed as previously described (60). The incidence of CFU-F (>50 cells) was determined and the data presented as mean number of CFU-F +/-SEM of triplicate cultures (statistics, ANOVA).

**Figure 2.** MSC numbers are elevated in primary stromal cultures from myeloma patients. Primary stromal cultures were established from STRO-1-selected BM MNC preparations derived from healthy donors (HD) (n=9) and myeloma patients (MM) (n=20). (A) and (B) Representative 2-colour flow cytometric dot plots of HD (A) and MM (B) stromal culture stained with an antibody to the MSC marker STRO-1 and the OB marker alkaline phosphatase (ALP). (C) Relative proportions of STRO-1+ALP- MSC and STRO-1-ALP+ OB in healthy donors (n = 9) and a patients with myeloma (n = 20) are presented as mean ± SEM (ANOVA).

**Figure 3.** The number of STRO-1+ALP- MSC correlate positively with plasma cell burden at the time of diagnosis. (A) Correlation analysis revealed a positive correlation between CD138+ PC number and STRO-1+ MSC numbers (R^2=0.4976, p<0.01, n=20, Spearman Rank) and (B) a negative correlation between PC number and ALP+ osteoblast numbers (R^2=0.3125, p<0.01, n=20, Spearman Rank).

**Figure 4.** Human STRO-1+ALP- MSC are a rich source of OC- and myeloma PC-activating factors. (A) Primary stromal cultures derived from healthy donors (n=3) were stained and sorted on the basis of their expression of STRO-1 (quadrant 1) or ALP (quadrant 3). (B) RNA was extracted from the STRO-1+ALP- and STRO-1-ALP+ sorted fractions and the relative expression of the PC and OC-recruitment and activation factors, RANKL, OPG and IL-6 was examined by real time PCR. Compared to STRO-1-ALP+ OBs (white bars), the STRO-1+ MSC (filled bars) express significantly higher levels of OC and PC recruitment/activating factors RANKL (p<0.05, t-test) and IL-6 (p<0.05, t-test), respectively. In contrast, the STRO-1-ALP+ OBs expressed significantly higher levels of the OC-inhibitory factor OPG (p<0.001, t-test). Data are presented as mRNA expression normalised to β-actin.
Figure 5. **MM disease progression is accompanied by changes in the BM mesenchyme in mouse model of MM.** Six week old C57BL6/KaLwRijHsd mice (n=8) were infused with 5x10^5 luciferase/GFP-labelled 5TGM1 myeloma PC. Age matched, control mice (n=8) were injected with vehicle alone (PBS). (A) Myeloma disease progression was monitored by bioluminescent imaging at weekly intervals. (B) BMMNC were recovered from control and tumour bearing mice at 2 and 4 weeks post injection. Cells were stained and sorted by FACS to eliminate the lin+CD45+CD31+ cells. The CFU-F potential of the remaining lin-CD45-CD31- was determined as described in the Methods. The data are presented as mean number of CFU-F +/-SEM of triplicate cultures (*p<0.05, t-test).

Figure 6. **Flow cytometric isolation of CB-derived MSC and OB.** Bones from C57BL6/KaLwRijHsd mice were gently crushed and rinsed with PBS to remove the BM. The bone fragments were digested with collagenase and the resultant single cell suspension depleted of mature haemopoietic-lineage cells using a cocktail of biotinylated antibodies to lineage markers as described in the Methods. (A) An example dot-plot displaying Lineage (Lin) versus CD45 fluorescence. (B) The non-haemopoietic lin-CD45- cells were further resolved based on their expression of CD31. (C) The CD31- fraction was further subdivided with CD51 and Sca1 to resolve 3 populations: the lin-CD45-Sca-1+CD51+CD31- OP, the lin-CD45-Sca-1-CD51+CD31- OB and the lin-CD45-Sca-1+CD51-CD31- MSC. (D) Schematic showing the acquisition/loss of cell surface molecules following MSC differentiation into OB. (E &F) Compact bone cells were isolated and the number of OB (E) and MSC (F) were enumerated by flow cytometry. The number of Sca-1-CD51+ OB were significantly decreased at week 4 between the tumour-bearing mice compared to the control mice (p<0.05, t-test). In contrast, the number of Sca-1+CD51- MSC was significantly increased in the tumour-bearing mice cell at 4 weeks (p<0.05, t-test).

Figure 7. **Murine MSC are a rich source of IL-6.** (A) RNA was prepared from freshly isolated lin-CD45-Sca-1+CD51+CD31- OB and the lin-CD45-Sca-1+CD51-CD31- MSC recovered from healthy C57BL6/KaLwRijHsd mice. (B) The relative expression of the PC and OC-recruitment and activation factors, RANKL, OPG and IL-6 was examined by real time PCR. Compared to the CD45-Sca-1+CD51+CD31- OB population, the lin-CD45-Sca-1+CD51-CD31- MSC population was an abundant source of IL-6 (p<0.05, t-test). Like the human MSC, the murine MSC expressed significantly less OPG compared with the OB population (p<0.05, t-test). Data are presented as mRNA expression normalised to β2M.
Fig 3

A

STRO-1 (%) vs. PLASMA CELL (%)

R² = 0.4976
n = 20
p < 0.01

B

ALK PHOS (%) vs. PLASMA CELL (%)

R² = 0.3125
n = 20
p < 0.01
Fig 6

A

Lin-APC

CD45 Alexa Fluor 780

B

FluoroGold

CD31 PerCP-Cy5.5

C

OB

OP

Sca1 PE-Cy7

MSC

D

Mesenchymal Stem Cell (MSC)

Sca-1+ CD51- CD31-

Osteoprogenitor (OP)

Sca-1+ CD51+ CD31-

Osteoblast (OB)

Sca-1- CD51+ CD31-

E

Osteoblasts (CD51+Sca1-)

% of CD45-Lin-Population

Week 2

Week 4

p<0.05

F

Mesenchymal Stem Cells (CD51-Sca1+)

% of CD45-Lin-Population

Week 2

Week 4

p<0.05

Control Tumour Control Tumour

Control Tumour
Fig 7

A

B

Gene Expression (relative to β-actin)

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<tr>
<td>IL-6</td>
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p < 0.05
Methods (supplementary):

**Colony Forming Unit-Fibroblast (CFU-F) Assay**

Unfractionated or immune-selected BMMNC were plated at densities ranging from 0.1 to 1 x 10^4 per cm^2 and grown in α-MEM supplemented with 20% fetal calf serum, 2 mM L-glutamine and 100 µM L-ascorbate-2-phosphate, 50 U/ml Penicillin, 50 µg/ml Streptomycin in 5% CO₂ at 37°C humidified atmosphere, as previously described (1). Colonies (cell clusters of >50 cells) were counted following fixation with 4% paraformaldehyde and staining with 0.1% toluidine blue. Primary BMSSC cultures were established by plating 1 to 5 x 10^4 unfractionated or STRO-1+ immune-selected BMMNC per cm^2 and then grown in α-MEM as described above.

**Enzymatic Digestion of BM Stromal Cultures**

Single cell suspensions were obtained from confluent primary stromal cultures by enzymatic digestion, as previously described (2). The cultures were washed twice in phosphate buffered saline pH 7.4 and then digested in a solution of collagenase (3 mg/ml) (Collagenase Type I; Worthington Biochemical Co., Freehold, NJ) and dispase (4 mg/ml) (Neutral Protease Grade II; Boehringer Mannheim, GMBH, Germany) for 90 minutes at 37°C. Cell suspensions were then washed with growth medium before being passed through a Falcon cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) to obtain a single cell suspension.

**Flow Cytometric Analysis and Cell Sorting**
Stromal cells were resuspended in blocking buffer (HBSS + 20 mM Hepes, 1% normal human AB serum, 1% bovine serum albumin (BSA: Cohn fraction V, Sigma Aldrich Pty Ltd, NSW, Australia), and 5% FCS for 20 minutes on ice. Approximately 1-3 x 10^7 cells were pelleted in 14 ml polypropylene tubes (Falcon; Becton Dickinson, Franklin Lakes, NJ) and resuspended in 200 ml of saturating concentrations of B4-7827 (mouse IgG1 anti-human bone/liver/kidney alkaline phosphatase; Developmental Studies Hybridoma Bank, Iowa University, IA) and STRO-1 for 45 minutes on ice. The isotype matched negative control antibodies, IgG1 (3D3) and IgM (1A6.12) were used under identical conditions. Cells were washed in HBSS with 5% FCS and incubated with a goat anti-mouse IgG-PE (1/50) and a goat anti-mouse IgM-FITC (1/30) (Southern Biotechnology Associates, Birmingham, AL) for 45 minutes on ice. Cells were washed a further two times and resuspended to approximately 10^6 cells/ml prior to analysis with a Beckman Coulter Cytomics FC500 (Beckman Coulter, Miami, FL) using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2, or sorting with a Becton Dickinson Aria flow cytometer (BD Biosciences, San Diego, CA), using FACS Diva Software version 6.1.3 and re-analysis using FCS Express for Flow Cytometry version 4 (De Novo Software, Los Angeles, CA). Positivity for each antibody was defined as the level of fluorescence greater than 99% of the isotype matched control antibodies. Following the initial sort each STRO-1/AP subpopulation was re-sorted and analysed to ensure a purity of >99%.

**Isolation of Compact Bone-Derived Cells**

Mice were humanely killed 4-weeks post tumour inoculation. Tibiae and femora were extracted and cleaned thoroughly. Bones were gently crushed with a mortar and
pestle and washed with 2% FCS/2 mM EDTA/PBS solution to remove marrow. Bones were incubated in 3 mg/mL collagenase solution containing 0.2% DNase for 5 minutes and cut finely using a #22 scalpel. Bone fragments were incubated in collagenase solution for 45 minutes, shaking at 37°C. Supernatant was collected through a 70 um nylon cell strainer and centrifuged at 400 x g for 10 minutes to collect cells.

**RNA Isolation and Real-time PCR**

For human samples, total cellular RNA was routinely prepared from 2 x 10^4 FACS double-sorted stromal cell subpopulations using Trizol extraction method (Life Technologies), as per the manufacturer’s protocol. 1-2 µg of RNA was reverse transcribed using Superscript III (Life Technologies) as per the manufacturer’s protocol. Real-time PCR was conducted on the Corbett Rotorgene using the primers detailed in Table 1. For mouse studies, RNA was extracted using the RNeasy Micro Kit, including DNase I treatment (Qiagen, Hilden, Germany) according to manufacturer’s protocol. cDNA was synthesised with random primers using the AffinityScript QPCR cDNA synthesis kit (Agilent Technologies, Victoria, Australia) according to manufacturer’s protocol. Real-time PCR was conducted using Brilliant II SYBR Green QPCR master mix (Agilent Technologies) in 20 µl reactions using 1 µl each forward and reverse primers (5 µM) on an Agilent Technologies, Stratagene MX3000P machine using primers detailed in Table 1.
### Table 1. Real-time PCR primers

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### References