THE ROLE OF MYELOPEROXIDASE IN MULTIPLE MYELOMA

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by

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ABSTRACT

Multiple Myeloma (MM) is an incurable haematological malignancy characterised by the uncontrolled outgrowth of clonal plasma cells and is preceded by the premalignant phase, monoclonal gammopathy of undetermined significance (MGUS). It is currently unknown what drives MGUS-to-MM transition; however, recent studies suggest exogenous factors within the bone marrow (BM) microenvironment play a pivotal role. Notably, MGUS has been associated with skeletal fragility, with an increased risk of fractures. Myeloperoxidase (MPO) is an enzyme abundantly deposited at sites of fracture by infiltrating neutrophils. Recent studies have elucidated novel roles for MPO in tumorigenesis, however it is yet to be implicated in the context of MM. We aimed to evaluate the role of MPO in driving MM progression, utilising *in vitro* proliferation and migration models, and the well-characterised C57Bl/KaLwRij/5TGM1 mouse model of MM.

In KaLwRij mice, induced intratibial damage with heightened MPO activity, enhanced 5TGM1 MM cell homing to the site of injury (p=0.0045). Interestingly, we show that MPO significantly upregulates the mRNA expression of major MM growth factors, IL-6 and VEGF in both human and murine BM stromal cells. Furthermore, MPO stimulated BM stromal cells significantly increased murine (p=0.000019) and human (p=0.0087) MM cell proliferation. Additionally, MPO promoted 5TGM1 cell migration *in vitro (*p=0.038) and induced cell signal transduction. Finally, in the KaLwRij/5TGM1 model, injected MPO significantly enhanced tumour burden within the tibia BM by 16% after 24 days (p=0.048). Collectively, these findings suggest that MPO may be a viable therapeutic target in limiting the progression from MGUS to active MM.

Word Count: 250

INTRODUCTION

Multiple Myeloma (MM) is an incurable haematological malignancy of the plasma cells (PC) within the bone marrow (BM)[1]. MM is the second most common haematological malignancy with a global 5-year prevalence of 230 000 patients with the median age of approximately 70 years[2,3]. MM is characterised by the dysregulation of monoclonal PC proliferation, consequentially resulting in the overproduction of immunoglobulins and immunoglobulin light chains which lack any known function[1,4]. MM diagnosis requires >10% of clonal plasma cells within the BM, accompanied by the presence of one or more features of end stage organ damage often referred to as 'CRAB' features (hypercalcemia, renal failure, anemia, lytic bone lesions)[5]. MM is preceded by a premalignant condition known as monoclonal gammopathy of undetermined significance (MGUS). MGUS is a disorder, characterised by <10% BM PC and the absence of the end stage organ damage seen in MM[6,7]. Current treatment of MGUS consists of a 'wait and see approach', which involves regular screening until evidence of MM features manifest. Patients with MGUS often maintain stable levels of clonal PC for extended periods of time. However, in approximately 1% of patients annually, MGUS progresses to MM due to unknown pathophysiological mechanisms[7,8]. Until recently, progression of MM from MGUS was thought to be a result of the gradual acquisition of driver mutations within the abnormal PC clones[6,9]. However, recent studies conducted by Dutta and colleagues, which utilized whole exome sequencing of PC samples collected from patients with MGUS who subsequently went on to develop MM, were successful in identifying that disease progression is predominantly associated with the outgrowth of PC clones already present at the MGUS stage[10]. Moreover, recent studies using transgenic mice engineered to express MM PC growth factors have shown that intrafemoral xenografts of PC from MGUS patients were as capable of engraftment as PC from symptomatic MM patients, suggesting that MGUS PC have the same proliferative potential as MM PC, if provided with a growth supportive BM microenvironment[11]. These findings suggest that rather than acquisition of new genetic mutations triggering the dysregulation of clonal PC proliferation, the

progression from MGUS to MM may be largely regulated by changes within the BM microenvironment.

A number of recent studies have identified that patients with MGUS often acquire skeletal fragility, coinciding with a substantially increased risk of bone fracture[12-14]. The fracture healing process is made up of a complex of regenerative physiological processes, with immune cells playing a pivotal role, in particular, neutrophils[15]. Neutrophils have been widely observed as the most abundant cell type in the early fracture hematoma[16]. Myeloperoxidase (MPO) is a heme containing peroxidase enzyme, expressed in abundance by neutrophils, constituting up 5% of the cells dry mass. In the presence of hydrogen peroxide (H_2O_2), MPO oxidizes chloride ions, to generate hypochlorous acid which has been shown to have potent antimicrobial properties[17, 18, 19]. Interestingly, neutrophil infiltration and accumulation within tumour sites has largely been associated with poorer patient prognosis and increased disease burden[20,21]. Recent studies have elucidated new roles for MPO on multiple cell types driving fibroblastic, osteoblastic, and vascular cell functions[22, 23]. Moreover, MPO has been shown to be overexpressed in patients with leukemia, as well as abundantly deposited in breast cancer [24, 25]. Strikingly, in a well-characterised mouse model of breast cancer, direct MPO stimulation was shown to have a causative role in driving breast cancer progression via regulation of the tumour microenvironment, with an increase in collagen deposition, vascularization, and a concomitant increase in lung metastasis[23].

Notably, IL-6 is highly upregulated in the serum of MM patients and to date has been well characterised as a significant growth factor for myeloma plasma cells[26]. Interestingly, IL-6 production is increased in older patients in response to fractures compared to their younger counterparts and is a key driver of MM pathogenesis[27]. In addition to its direct proliferative effects on MM cells, IL-6 acts through aiding in the progression of the disease through enhancing the vascularization within the bone marrow[28]. IL-6 is also is known to promote angiogenesis in MM through the enhancement of

vascular endothelial growth factor (VEGF)[29]. Enhanced vascularity is considered a hallmark of MM and has been has been well characterised as a negative prognostic factor[30]. Furthermore, MPO was recently identified as a driver of angiogenesis with reported increases in expression of both VEGF and IL-6 in human endothelial cells[22]. Therefore, MPO deposited at sites of fracture may play a role in regulating the bone marrow microenvironment, subsequentially creating a niche which facilitates the outgrowth of MGUS plasma cells, and drive transition to active MM.

HYPOTHESIS AND AIMS

We hypothesize that increased deposition of myeloperoxidase by inflammatory cells at sites of bone damage and/or fractures creates a permissive tumor microenvironment for MGUS plasma cells to thrive and progress to active MM:
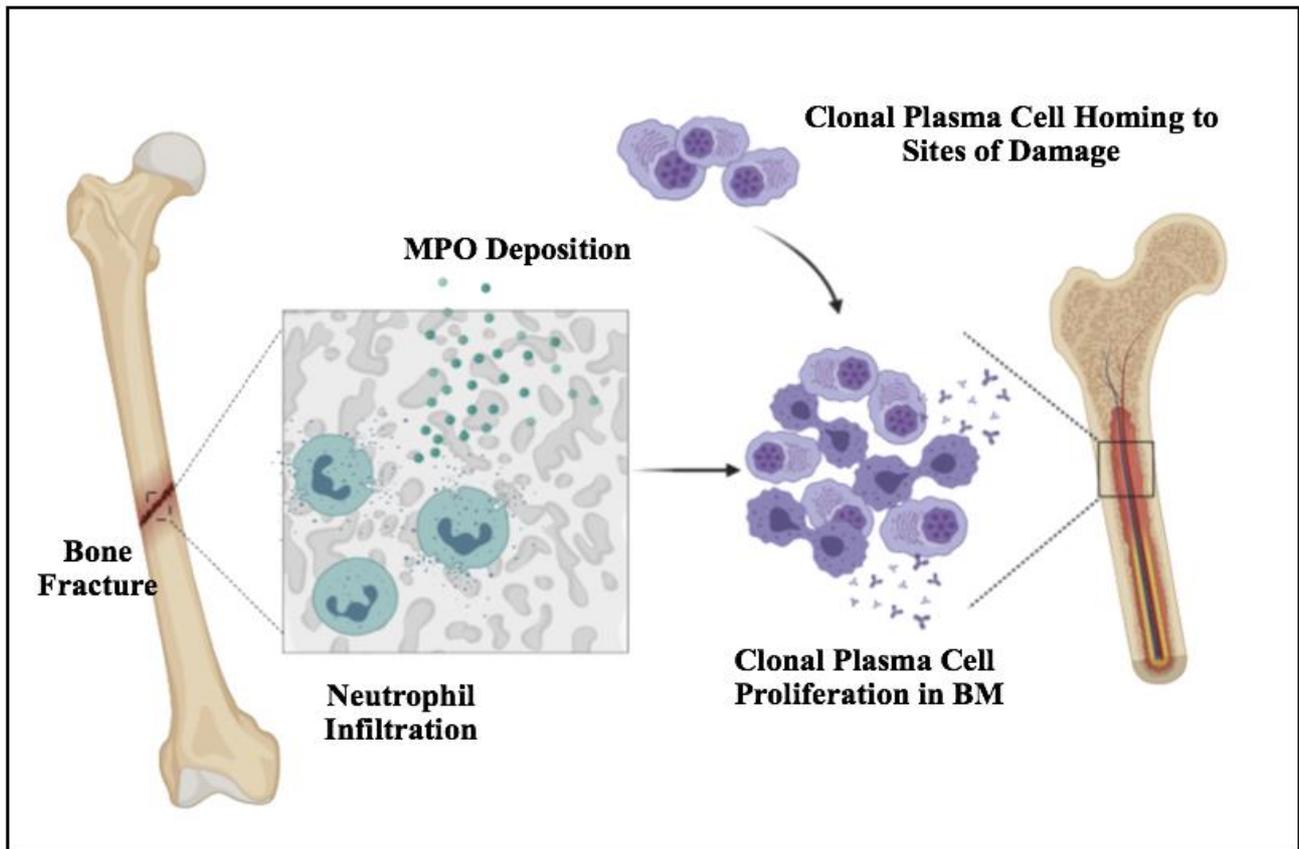
Figure 1. Hypothesis regarding MPO deposition within sites of bone fracture

This hypothesis will be addressed through the following aims:

1. To determine whether induced inflammation with an increase in MPO activity within the bone marrow increases MM homing.

2. To determine the role of MPO on MM proliferation and migration *in vitro* and examine the mechanisms and signaling pathways involved

3. To investigate the effect of MPO deposition in MM development and progression using *in vivo* models of MM.

MATERIALS AND METHODS

Cell Culture

All tissue culture reagents used were manufactured by Sigma Aldrich (St. Louis, MO, USA) unless otherwise specified. Cells were cultured under sterile conditions, maintained at 37˚C in the presence of 5% CO_2. All media was supplemented with 10,000 U/mL penicillin, 10 mg/ml streptomycin, 200 mM L-glutamine, 100 mM sodium pyruvate and 1 mM HEPES. Murine 5TGM1 myeloma cell line was cultured in complete Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% foetal calf serum (FCS). Human OPM-2 myeloma cell line was maintained in Roswell Park Memorial Institute Medium (RPMI-1640), supplemented with 10% FCS. Murine OP9 stromal cells and Human Osteoblast (NHB) stromal cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) – high glucose supplemented with 10% FCS.

Animals

Age and sex matched C57Bl/KaLwRij mouse were housed at the South Australian Health and Medical Research Institute (SAHMRI) within Bio-resources under pathogen free conditions. Experimental work and intervention were carried out in accordance with the University of Adelaide and SAHMRI ethics.

Mouse Models of MM

Acute tibial injury and direct recombinant MPO injection (10µg) was achieved via insertion of a 26-gauge intubator into the medullary cavity. To detect MPO activity, mice were injected with 125µL of luminol (40mg/mL in PBS, Sigma) and imaged using an IVIS Spectrum imaging system (Caliper, Almameda, CA). For intravenous (i.v.) model, $5x10^6$ luciferase-expressing GFP+ 5TGM1 cells, resuspended in 100µL of PBS were injected through the tail vein of 6-8 week old C57Bl/KaLwRij. Mice were subject to regional hindlimb BLI, in addition to endpoint flow cytometry of GFP+ tumour cells in complete marrow harvested from tibias as previously described[31]. To asses tumour burden,

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mice were administered with firefly D-Luciferin (30 mg/ml in PBS, Biosynth). Mice were subject to regional hindlimb BLI. At the study endpoint, complete marrow was harvested from tibias and analysed for GFP+ tumour cells via flow cytometry as previously described[32].

Transwell Migration

MM PC migration was determined *in vitro* using 24 well transwell plate (BD Falcon FluoroBlok™) system, encompassing PET membranes with 8.0µm pores (n=2). GFP+ 5TGM1 cells were serum-starved in 1% FCS IMDM for 1 hour prior to 5×10^5 cells in 100 µL being transferred into the upper chamber of the transwell. 600 µL of normal cell culture media at varying concentrations of MPO (0.25 µg/mL, 0.5 µg/mL, 1 µg/mL) was added to the lower chamber. Cells were allowed to migrate for 24 hr with 20% FCS IMDM culture media acting as the vehicle control. Migrated GFP+ 5TGM1 cells were photographed and quantified using a fluorescent microscope as previously described[33].

RNA Isolation & Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Murine OP9 and human NHB BM stromal cells were seeded into 6 well plates and were cultured in serum-free DMEM cell culture media acting as the vehicle control or supplemented with Myeloperoxidase at indicated concentrations (n=1). Total RNA was extracted using TRIzol (Life Technologies, Calsbad, CA, USA) after 24 hours. After isolating RNA, Superscript IV was used to synthesize cDNA as previously described.[34] Quantification of gene expression was achieved through RT-PCR using SYBR-Green Fluor qPCR mastermix on a Biorad CFX Connect as previously described.[23] Samples were run in triplicate, with gene expression presented as fold change compared to the vehicle control. Primer pairs: human IL6 5'- ACAGACAGCCACTCACCT-3' (forward), 5'-TTTCACCAGGCAAGTCTCCT-3' (reverse); human VEGF 5-ATGCCAAGTGGTCCCAGG-3' (forward), 5'-CACACAGGATGGCTTGAAGA-3' (reverse); human β-actin 5'-GATCATTGCTCCTCCTGAGC -3' (forward) 5'- GTCATAGTCCGCCTAGAAGCAT-3' (reverse); murine *Il6* 5'- GCCTTCTTGGGACTGATGCT-3' (forward) 5'-

CTGCAAGTGCATCATCGTTGT-3' (reverse); murine *Gapdh* 5'-TGCACCACCAACTGCTTAG-3' (forward) 5'-GGATGCAGGGATGATGTTC-3' (reverse).

IL-6 Enzyme Linked Immunosorbent Assay (ELISA)

To evaluate the effect of MPO stimulation on stromal cell production of IL-6, human stromal cells were seeded into 96 well plates at a density of $1x10^4$ cells/well in 100 µl 10% FCS DMEM and were allowed to culture for 5 days until reaching confluence. Media was removed and cells were stimulated for 24, 48 and 72 hr in serum-free DMEM containing MPO, or serum-free DMEM cell culture media acting as the vehicle control. At the end of each stimulation period, supernatant was collected to allow the measurement of soluble IL-6 by ELISA. The amount of soluble IL-6 in the supernatant was measured using a Quantikine ELISA Human IL-6 Immunoassay (R&D Systems, Inc. USA), as per manufacturer's instructions, using a standard curve formulated from recombinant human IL-6.

In Vitro Conditioned Media - MM Proliferation

Murine OP9 or human NHB stromal cells (n=2) were seeded ($2x10^5$) into T25 cell culture flasks and were allowed to grow to confluency in 10% FCS DMEM, with media changes 2-3 times per week. Stroma was then stimulated with recombinant human MPO at increasing concentrations (0.25 µg/mL, 0.5 µg/mL, 0.75 µg/mL, 1 µg/mL, 1.5 µg/mL) in 2 mL of 1%FCS IMDM for 48 hr, 1% FCS IMDM served as the vehicle control. Conditioned media was then collected and mixed at a ratio of 50:50. 5TGM1 cells were seeded in 20% FCS IMDM final, in corresponding OP9 conditioned media, whilst OPM2 cells were seeded in 10% FCS RPMI final in corresponding NHB conditioned media for 24 and 72 hr at 37°C and 5% CO_2. WST-1 (Roche) assay was used to quantify 5TGM1 proliferation as previously described[35].

Immunohistochemistry

IHC staining for MPO and GFP was performed on mouse formalin fixed paraffin embedded femurs.

Samples were subjected to heat-induced antigen retrieval (10 mM citrate buffer (pH 6)) at 97°C for 30 minutes, followed by endogenous peroxidase inactivation for 30 mins at room temperature using 0.5% H_2O_2 in methanol. To prevent non-specific binding of antibodies, slides were incubated with 3% normal goat serum 0.2% bovine serum albumin in PBS for 1 hr. Sections of tissue were incubated with primary antibodies (MPO (GA511, DAKO, Ready-to-use); and GFP (A6455, ThermoFisher, 1:1000) overnight. The following day slides were incubated with either EnVision/FLEX (DAKO, GV800;MPO) or anti-rabbit IgG biotin (Vector Labs, BA-1000;GFP) for 30 mins, followed by 1hr incubation with streptavidin-HRP (Vector Laboratories;GFP). Slides were developed using DAB (Vector Laboratories), counterstained with haematoxylin, and visualised using a light microscope (Eclipse 90i; Nikon).

MPO Immunofluorescence

Murine 5TGM1 myeloma plasma cells were seeded at a density of $5x10^5$ cells/ well into a 6 well plate and stimulated with MPO (2 µg/mL) for either 20 min or 3 hr. Cells were resuspended in 100 µL of neat FCS and placed into a cytocentrifuge to be spun onto slides for staining. NHB human stromal cells stimulated with MPO were used as the positive control. Each slide was washed 3x with PBS, before designated cells (Vehicle Control/ 3-hour timepoint) were permeated with 0.25% Triton X-100 in 1xPBS for 10 min. Slides were washed prior to blocking of nonspecific binding sites with 3% BSA in PBS and a 1:10 dilution of non-immune goat serum for 90 min at room temperature. Slides were then incubated with primary rabbit anti-MPO antibody (Bioss Inc. Boston Massachusetts, USA) 1:100 dilution containing 1% BSA for 60 min at room temperature. Labelled proteins were then detected with 1:500 dilution of anti-rabbit IgG AlexaFluor594 in 1% BSA in PBS in the dark for 60 min at room temperature. A Leica TCS SP8X confocal microscope was used to capture fluorescence.

Western Blot Analysis

Murine 5TGM1 myeloma plasma cells were seeded into T25 cell culture flasks and starved in serum-free IMDM (0.5% FCS) overnight and then stimulated with indicated treatments for 5, 15 and 30 min. Lysates were prepared as previously described[36]. Briefly, 50 µg of each lysate were separated using a 10% sodium dodecyl sulphate-polyacrimide gel, subjected to SDS-PAGE. Gels were transferred onto polyvinylidene difluoride membrane (PVDF, GE Healthcare). Immunoblotting was conducted using Anti-Phosphotyrosine clone 4G10® antibody (Millipore, Cat. No. 05-321, 1:1000 dilution). α-Tubulin acted as the loading control. Protein signal was visualised using enhanced chemifluorescence detection on an Odyssey CLx imaging system (LI-COR Bioscience, Lincoln, NE, USA).

Data Analysis.

Statistical analyses were conducted using GraphPad Prism (Version 8 GraphPad Software Inc, CA, USA) and Image J. Data was analysed using Students unpaired t-test (two-sided). $p < 0.05$ was deemed to be statistically significant.

RESULTS

Myeloperoxidase promotes homing of murine MM PC *in vivo*.

A striking feature of multiple myeloma is the ability of clonal MM PC to migrate and home to multiple bone microenvironments[37]. To evaluate whether inflammatory MPO deposition promotes myeloma plasma cell homing to the bone marrow, a localised inflammatory response was induced by performing a sham intratibial (i.t.) injection in the left tibia of male C57Bl/KaLwRij mice 24 hr prior to 5TGM1 tumour cell inoculation. The increase in localised MPO activity within the tibia was confirmed using luminol reagent (Fig 1A). Flow cytometry analysis of the harvested bone marrow revealed a significant increase (p=0.0045) in the number of GFP+ 5TGM1 MM cells present in the Sham i.t. tibia compared to the contralateral control tibia (Fig. 1B).
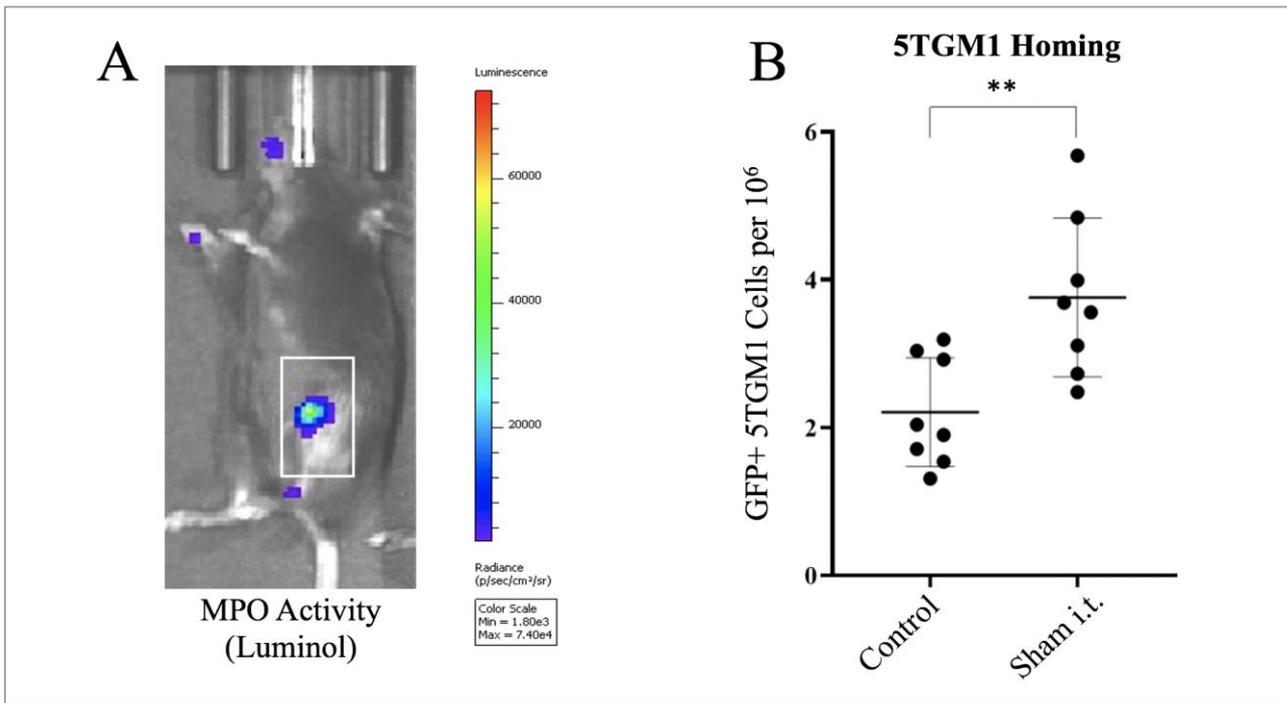
Figure 2. Effect of increased MPO activity on murine 5TGM1 plasma cell homing. (**A**) Sham i.t. was performed in C57Bl/KaLwRij mice. Representative BLI (luminol) indicating MPO-activity at site of damage. (**B**) C57Bl/KaLwRij mice were injected with 5×10^6 GFP+ 5TGM1 cells in 100 µL of PBS via i.v. Flow cytometry was conducted on complete marrow from mice after 24 hr from the sham i.t. tibia in addition to the contralateral control leg. Quantified as total number of GFP+ cells per 1×10^6 cells. Results are shown as the mean ± SEM, n=8 mice. Statistical significance determined using unpaired student t-test, **p<0.005.

MPO promotes migration of MM PC in vitro.

To further examine the homing abilities of MPO in MM, we performed 5TGM1 plasma cell migration assays *in vitro*. GFP+ 5TGM1 cells were seeded in the the upper chamber of a BD Falcon FluoroBlok^{TM} transwell insert, with increasing concentrations of MPO in normal culture media in the lower chamber. After 24 hours, 5TGM1 migration was significantly increased 4 fold toward MPO at 0.25 µg/mL (p=0.038) when compared to the vehicle control (Fig. 1A.)
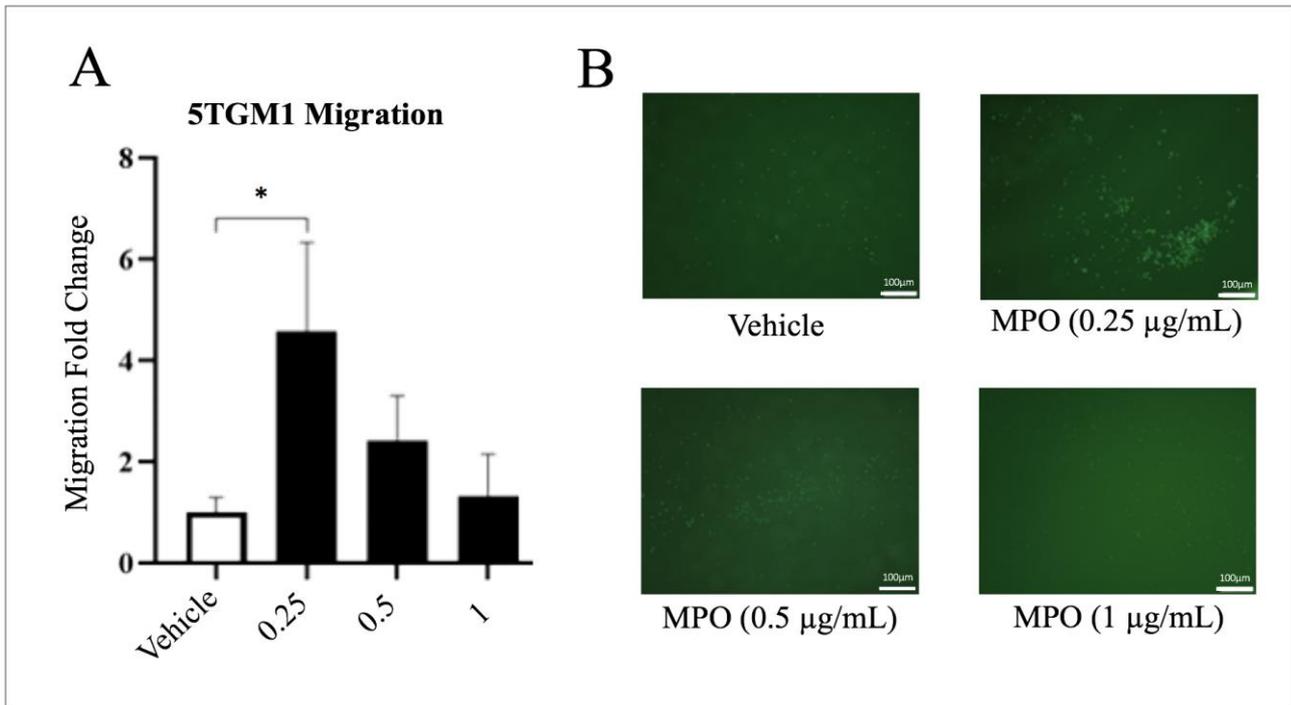
Figure 3. MPO independently promotes transwell migration of 5TGM1. (**A**) Transwell migration of GFP+ 5TGM1 cells after 24 hr. (**B**) Representative images of migrated GFP+ 5TGM1 cells. Cells were serum-starved in 1% FCS IMDM for 1 hr prior being seeded in the upper well of a transwell system. Migrated cells were photographed at 20x magnification and quantified using a fluorescent microscope. Scale bar 100µm. Mean ± SEM performed in triplicate, n=2. Statistical significance determined using unpaired student t-test, *p<0.05

MPO upregulates protumorigenic factors in bone marrow stromal cells.

MPO has previously been shown to regulate stromal cell gene regulation[23]. As MM is reliant upon the stroma for survival and proliferation, we investigated whether MPO stimulated the upregulation of two major growth factors involved in MM progression, *IL-6* and *VEGF*. Cultured murine OP9 and human NHB cells were stimulated with MPO at the concentrations indicated (Fig. 2A-B). RT-qPCR confirmed that MPO resulted in a maximal 13.1-fold (p=0.028) and 3-fold (p=0.033) increase in *IL-6*, with an 8-fold (p=0.000051) and 2.4-fold (p=0.0016) increase in *VEGF* mRNA expression in OP9 and NHB cell populations respectively. Furthermore, to determine whether the increase in *IL-6* expression exhibited in human NHB cells translated to protein synthesis, we performed an ELISA specific to human *IL-6*. We show that MPO at 0.5 µg/ml significantly increased soluble *IL-6* secreted by NHB stromal cells after 24 hours when compared to unstimulated control cultured (Fig. 2C) (p<0.05).
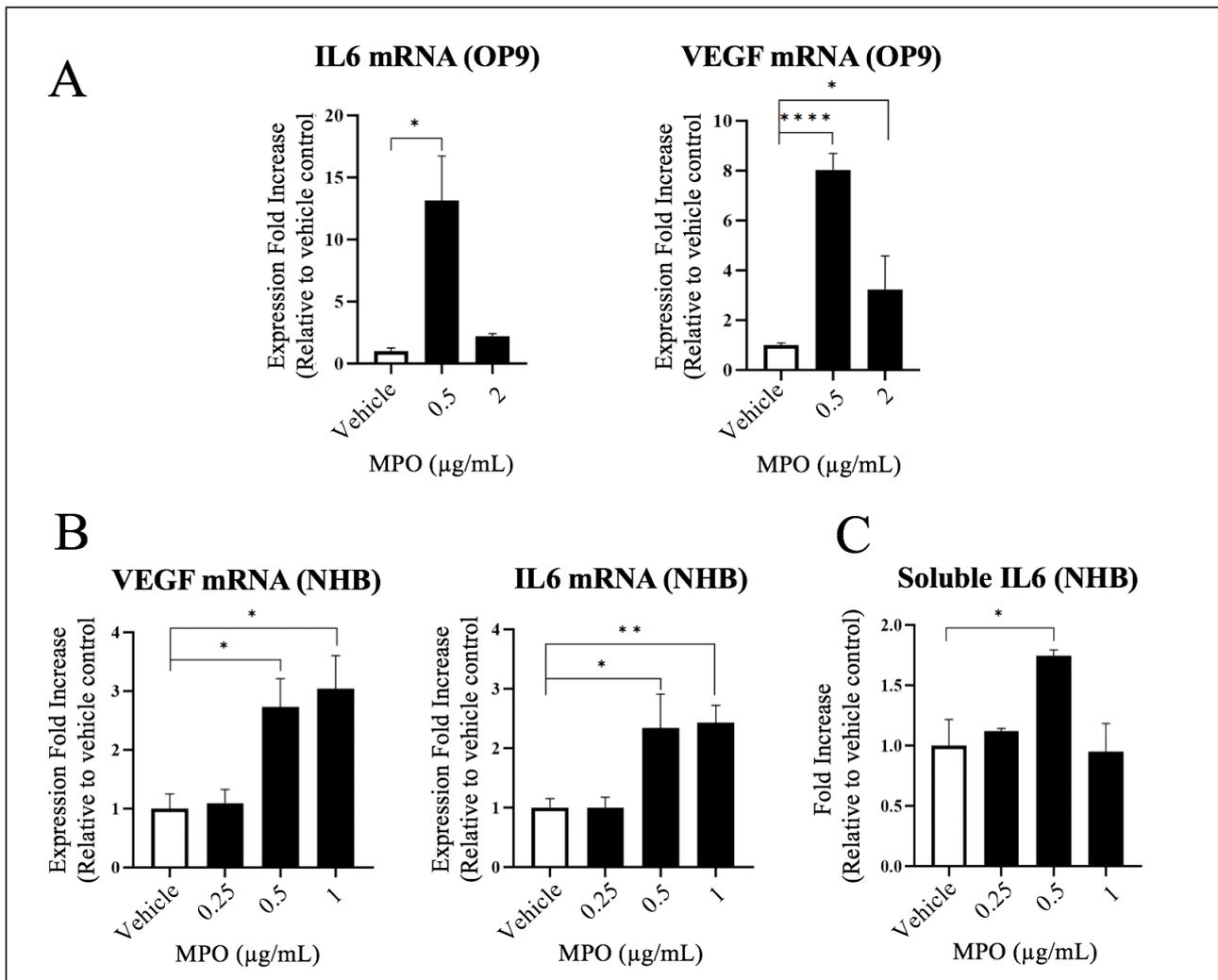
Figure 4. Effect of MPO stimulation on mRNA expression of IL-6 and VEGF in murine OP9 and human NHB stromal cells. RT-qPCR analysis of mRNA *IL-6* and *VEGF* expression in murine OP9 (**A**) and human NHB (**B**) stromal cells. Gene expression was measured following 24 hr culture in the presence of MPO at the concentrations indicated. Normalised to *Gapdh* and *β-actin* respectively. (**C**) Soluble IL-6 produced by NHB cells in response to MPO after 24 hours, quantified using ELISA detection. Expressed as fold change relative to serum-free DMEM vehicle control. Mean ± SD, performed in triplicate, n=1, previosuly confirmed in other cell populations. Statistical significance determined using Student's t-test, *p<0.05,**p<0.005, ****p<0.00005.

MPO promotes MM cell proliferation indirectly through interactions with BM stroma *in vitro*.

There exists a complex interaction between MM tumour cells and the bone marrow stromal cells, and exposure to stromal cells enhances MM disease progression[38]. To explore the indirect role of MPO in driving myeloma plasma cell proliferation through interactions with the BM microenvironment, we investigated the proliferation of murine 5TGM1 and human OPM2 myeloma plasma cell lines *in vitro* supplemented with MPO-treated conditioned media. Conditioned media was generated by stimulating murine OP9 or human NHB cells in reduced-serum DMEM supplemented with MPO at varying concentrations for 48 hr prior to being collected. Proliferation 5TGM1 ($p=0.000019$) and OPM2 ($p=0.0087$) cell lines were significantly increased after 72 hr in the presence of MPO stimulated stromal cell conditioned media (Fig. 5).
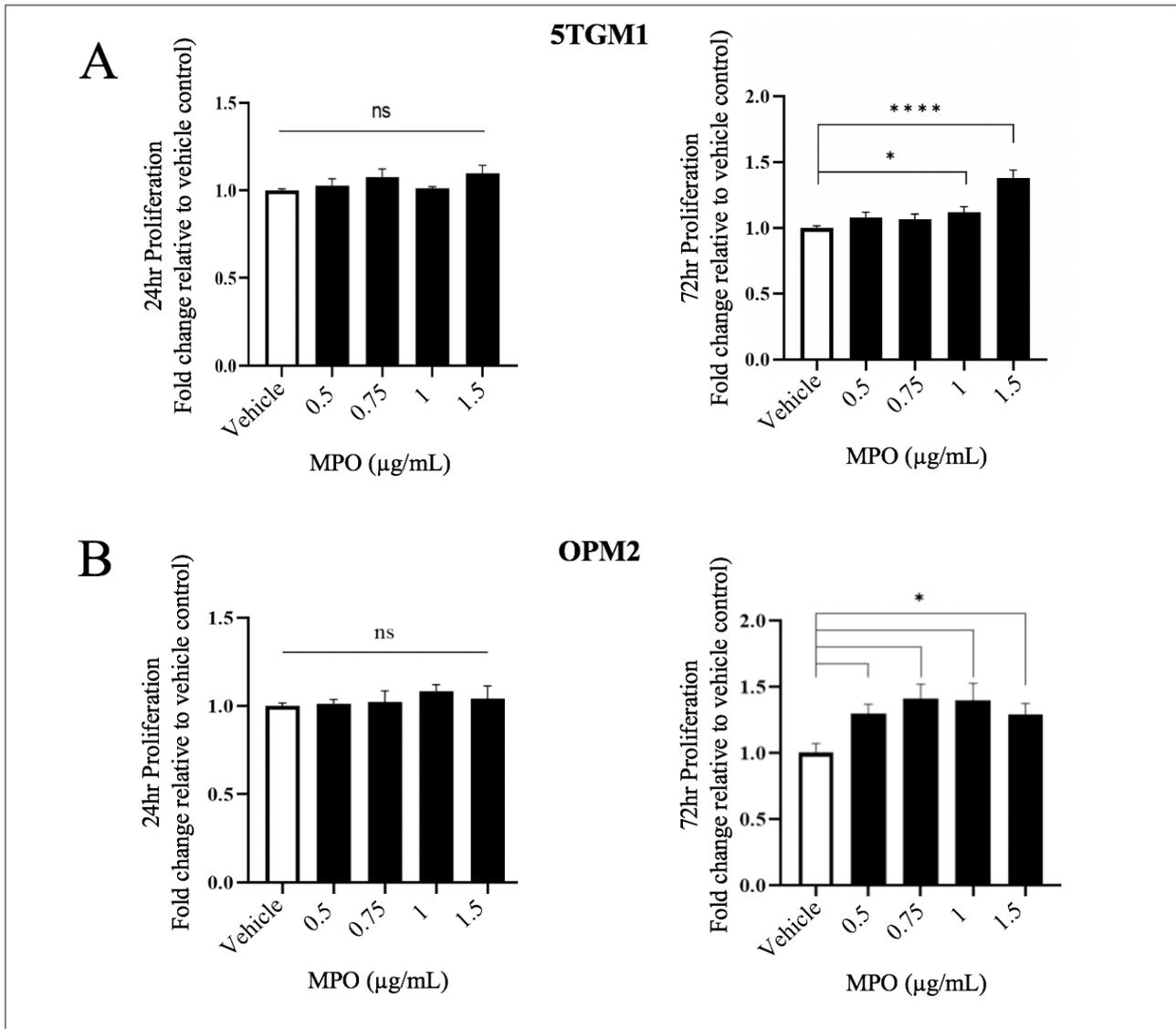
Figure 5. Proliferation of murine 5TGM1 and human OPM2 myeloma plasma cells in response to supplementation with MPO stimulated BM stromal cell conditioned media. Proliferation after 24 hr and 72 hr of murine 5TGM1 **(A)** and human OPM2 **(B)** cells cultured in supernatant collected from OP9 or NHB cells respectively, stimulated with MPO for 48 hr. Cells were incubated for 2 hr with addition of WST-1 reagent prior to quantification using colorimetric detection. Expressed as fold increase relative to the unstimulated control. Mean ± SEM performed in quadruplicate, n=2. Statistical significance determined using unpaired student t-test, *p<0.05, ****p<0.0005

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MPO is present and active at sites of MM

To investigate whether MPO is deposited within the tumour microenvironment in active MM, C57Bl/KaLwRij mice injected with luciferase-expressing 5TGM1 cells were subject to regional hindlimb BLI. MPO activity was detected through the use of luminol where it was found to be colocalized with sites of tumour. Furthermore, immunohistochemistry was performed on tissue sections of femurs isolated from C57Bl/KaLwRij mice injected with GFP+ 5TGM1 cells via i.v. Expression analysis of tumour burden femurs indicated strong expression of MPO, colocalized to expression of GFP.
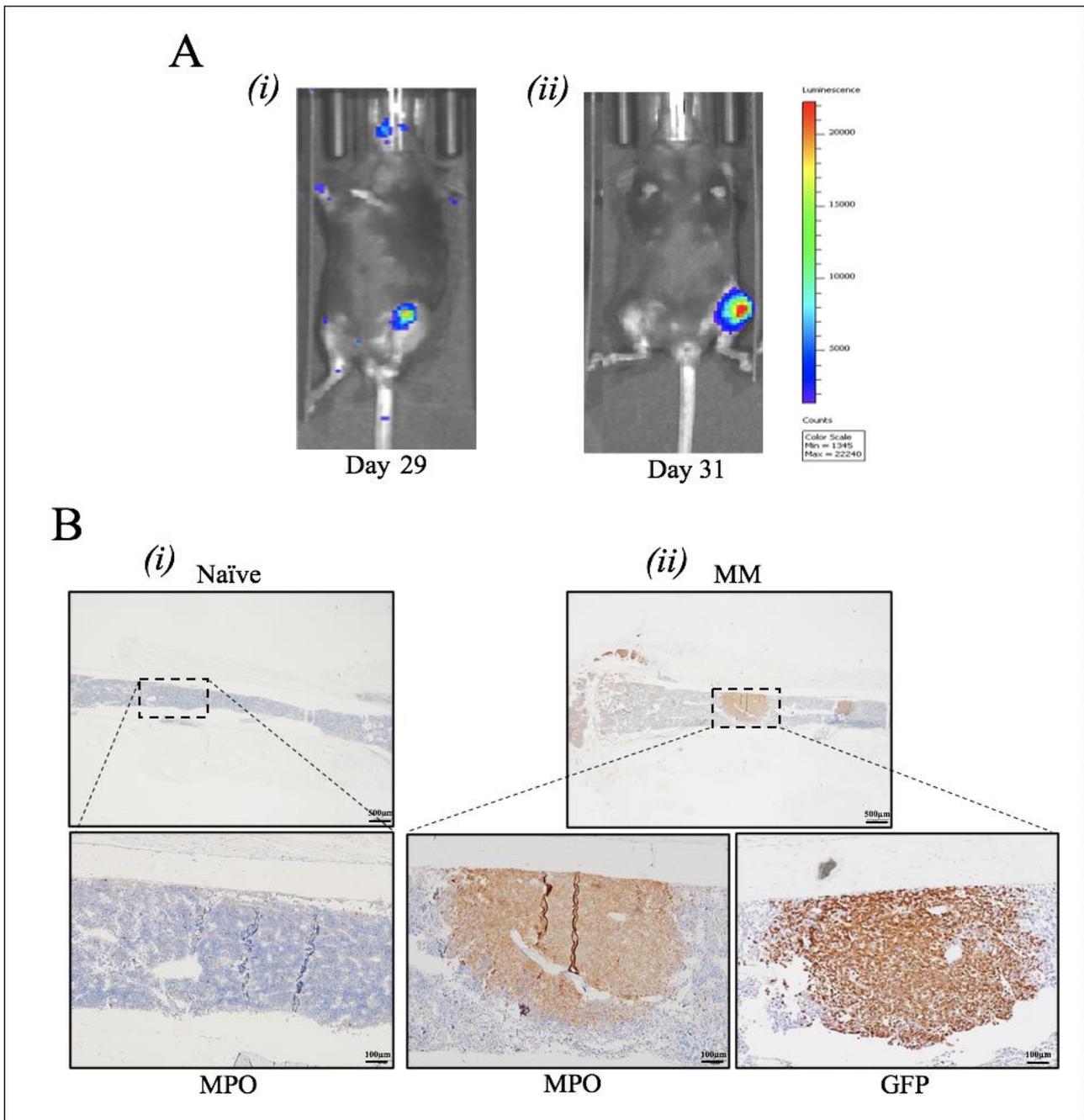
Figure 6. MPO expression is colocalized with 5TGM1 MM tumour in KaLwRij mice. **(A)** Representative BLI images indicating MPO activity indicated through luminol *(i)* and 5TGM1 tumour *(ii).* **(B)** Immunohistochemistry of femur sections taken from tumour naïve KaLwRij mice *(i)* or injected with 5TGM1 GFP+ cells via i.v. *(ii)*, stained with MPO. Representative image of GFP indicating colocalization of MPO and GFP+ 5TGM1 cells. Images taken at 4x and 10x magnification. Positive signal indicated as dark brown staining.

MPO readily binds to the cell surface and is internalised by 5TGM1 cells, activating phosphotyrosine signaling pathways.

To determine whether MPO interacts directly with MM plasma cells and mediate any potential mechanisms, murine 5TGM1 myeloma plasma cells were stimulated with MPO (2 µg/mL) for 20 min or 3 hr. Immunofluorescence studies showed that MPO readily binds to the surface of 5TGM1 cells within min (Fig 5A; Surface), as well as being taken up intracellularly after 3 hr (Fig 5A; Intracellular). No fluorescence was evident in the vehicle control, confirming the specificity of the MPO immunostaining. Furthermore, MPO stimulated phospho-tyrosine signalling within 5TGM1 plasma cells when compared to the untreated control (Fig. 5B). α-Tubulin served as the loading control.
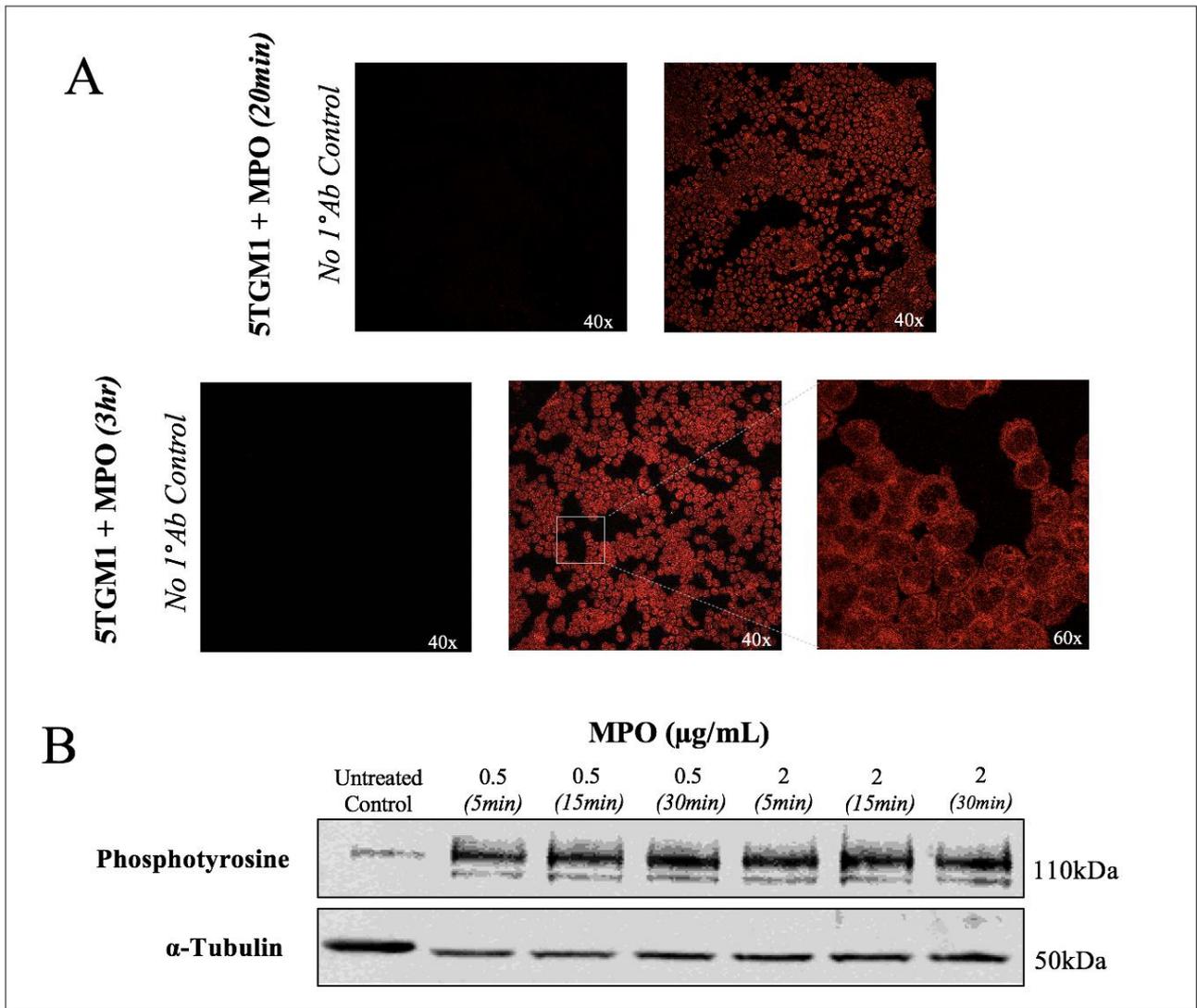
Figure 7. MPO directly interacts with murine 5TGM1 cells in vitro, activating signalling pathways involved in tyrosine phosphorylation. (**A**) Cultured 5TGM1 cells were stimulated with MPO for either 20 min or 3 hr and transferred to slides with a cytospin centrifuge. Cells were fixed, permeabilized (intracellular only) and stained for MPO localization. Surface images (40x), intracellular (60x) (**B**) Cultured 5TGM1 cells were stimulated with MPO for 5, 15 and 30 min at the concentrations indicated. Cell lysates were prepared and then subjected to western blot analysis with antibodies against pan-phospho-tyrosine residues and α-Tubulin.

Direct intratibial injection of MPO promotes proliferation of 5TGM1 cells *in vivo*.

To evaluate the role of MPO in MM progression, a luciferase-expressing, GFP+ murine 5TGM1 myeloma plasma cell line was utilized in the C57Bl/KaLwRij animal model of MM. Mice were injected with MPO (10 µg) via intratibial injection. After 72 hours, 5TGM1 cells were administered intravenously. Tumour progression was monitored weekly using BLI. Tumour burden was quantified through luciferase intensity (photons/second). Although statistical significance was not detected using BLI (Fig.8B), a positive trend is observed in the MPO injected leg compared to the contralateral leg after 24 days (p=0.10). Flow cytometry conducted on complete tibia marrow collected at day 27 indicated a significant increase in %GFP+ cells in MPO injected tibias when compared to the contralateral control (p=0.048).
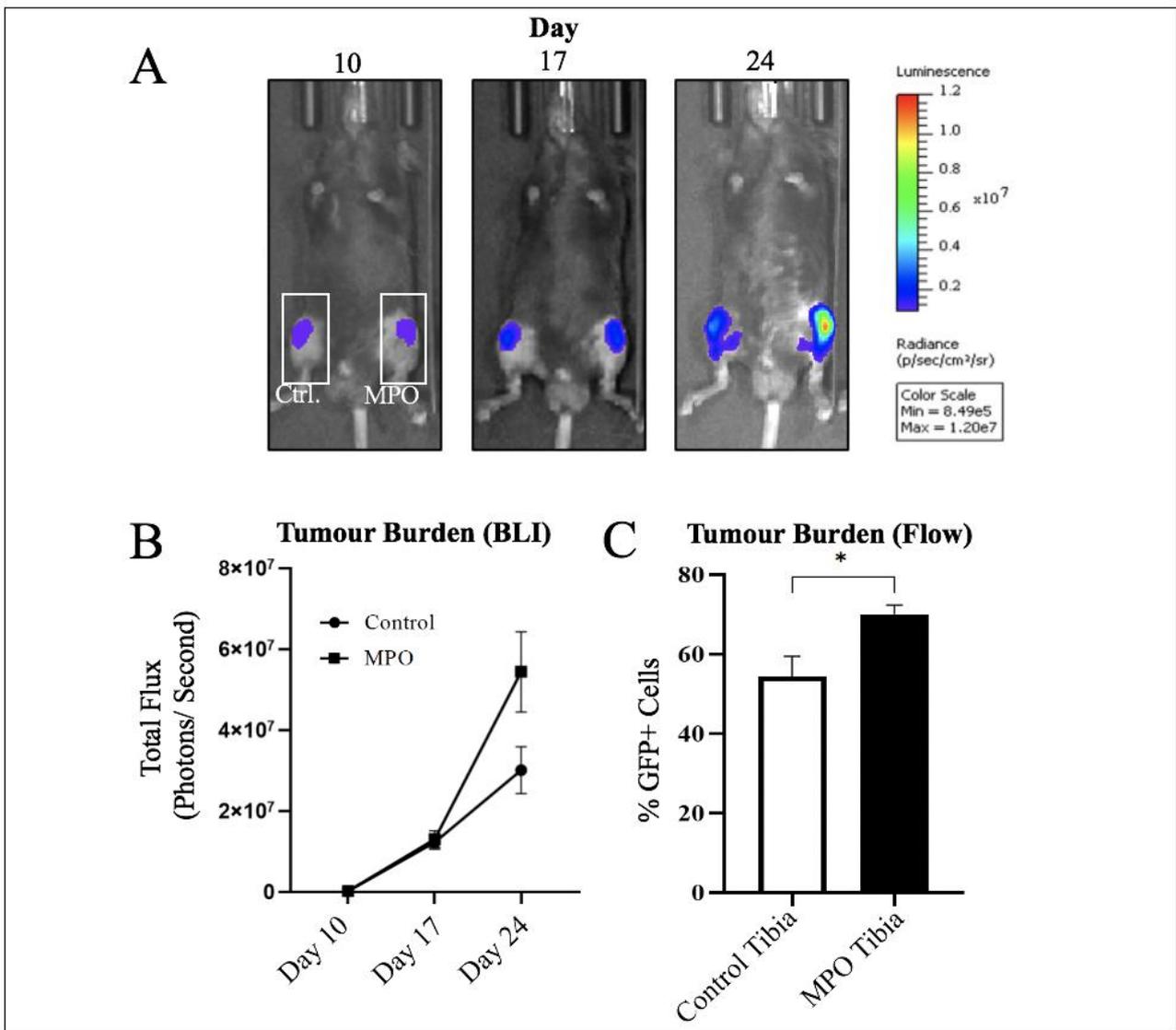
Figure 8. Effects of direct IT injection of MPO on 5TGM1 tumour progression in KaLwRij mice.
(**A**) Representative BLI images. MPO was administered via IT injection (10μg), with cells injected IV 72 hours post IT. Tumour burden expressed as total flux (photons per second) using BLI (**B**). Flow cytometry was conducted on complete marrow collected at day 26 from the MPO injected tibia and to the contralateral tibia which acted as the control. Quantified as percentage of 5TGM1 GFP+ cells (**C**). Mean ± SEM, n=3 mice. Statistical significance determined using Student's t-test, n=3 mice, *p<0.05.

DISCUSSION

The studies described herein provide exciting new data contributing to our ever-increasing understanding of the complex interplay between MM cells and the bone marrow microenvironment. Inflammation has been shown to play a critical role in the pathogenesis and progression of multiple cancer types, however, little is known about the interaction between inflammation and MM disease[39, 40]. The present study demonstrates that inflammatory MPO may play a causative role in MM progression. To date, it is currently unknown what drives the shift causing premalignant MGUS patients to progress to active MM disease. Recent findings have identified that exogenous factors and alterations within the bone marrow environment are likely to play a pivotal role in driving this transition[10, 11]. With recent studies associating MGUS with skeletal fragility, interest surrounds how deposition of inflammatory MPO at sites of fracture may influence MGUS plasma cells and drive progression. MPO has been recently reported to drive extracellular matrix production and angiogenesis, processes well characterised in tumorigenesis[22, 23, 34].

The first aim of this study was to investigate the homing capacity of MM cells to bone with increased inflammation and MPO activity. We show that in KaLwRij mice, murine 5TGM1 plasma cell homing is significantly increased to sites of damage caused by a sham i.t. injection after 24 hours. Interestingly, a population based study with over 14,000 MM patients revealed that MM patients with bone fractures at the time of MM diagnosis are at a 28% higher risk of succumbing to the disease[41]. Furthermore, our *in vitro* migration data shows that MPO can independently stimulate 5TGM1 migration. This finding coincides with other studies reporting the migratory effects of peroxidase enzymes, including MPO on fibroblasts, endothelial cells, and breast cancer cells[22, 23]. These results suggest that MPO has the capacity to increase the homing and migratory mechanism of MM cells and the likelihood of MGUS plasma cells migrating to sites of fractures, where they are susceptible to further outgrowth.

Multiple myeloma plasma cells rely upon stromal cell contact and soluble factors released by the stromal microenvironment for their survival, growth, and progression. Studies by Panagopoulos and colleagues previously reported that MPO promotes the progression of breast cancer through regulation of the stromal microenvironment[23]. Therefore, the second aim of this study was to investigate how MPO interacts with the stroma to promote MM progression *in vitro*. We identified that expression of VEGF, the primary growth factor for endothelial cells, and an essential contributor to vascularisation was significantly upregulated in both human and murine BM stromal cell populations in response to stimulation with MPO[42]. Taken collectively, our findings are consistent in highlighting the ability of MPO to independently promote angiogenesis as formerly described, creating a permissive microenvironment for MM cell expansion[22]. Moreover, in numerous malignancies, including MM, high serum concentrations of IL6 have been associated with poor patient prognosis[28, 43]. MPO stimulation increased production of soluble IL-6, an inflammatory cytokine well characterised to drive MM through both modulation of VEGF as well as promoting MM plasma cell proliferation directly[28, 44]. Furthermore, we were successful in implicating MPO does indeed drive MM cell proliferation indirectly through interactions with stroma *in vitro* using both murine and human cell populations. However, direct MPO stimulation failed to show any proliferative effects on MM cells (data not shown). Therefore, MPO's effects on MM proliferation is due to manipulating the stroma to create a permissive niche in which MM cells can thrive.

Given our IHC findings which identified MPO to be abundantly present at sites of myeloma, we further showed that MPO is readily taken up and internalised by MM cells, activating signalling pathways involved in the phosphorylation of tyrosine residues. While there exists a vast number of molecular pathways in MM cells, using a pan-phosphotyrosine antibody, we identified that MPO profoundly activates this signal transduction pathway[45]. A number of signalling pathways implicated in tyrosine phosphorylation have been well described in poor patient prognosis of MM such as

ERK1/2 and JAK/STAT, with further exploration needed to identify whether this interaction may be implicated in the context of MM progression[46, 47].

The final aim of this study was to determine whether MPO directly released within bone marrow microenvironment could promote MM progression. Direct injection of MPO into the bone marrow space before tumour cell inoculation significantly increased myeloma tumour burden by 16% compared to the contralateral tibia after 24 days. Rymaszewski and colleagues have previously indicated specific inhibition of the catalytic function of MPO, resulted in a reduced tumour burden of up to 50% in a murine model of lung carcinoma, suggesting inhibition of MPO may prove a viable complimentary therapeutic in limiting MM progression[48].

Collectively, our findings demonstrate that MPO has the potential to play a functional role in driving MGUS to MM transition through interactions with the bone marrow microenvironment and potential direct effect in myeloma cells themselves. The findings in the present study identifies MPO as a potential therapeutic target whereby inhibition of MPO activity may limit the transition into active disease.

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REFERECNCES

1. Gerecke C, Fuhrmann S, Strifler S, Schmidt-Hieber M, Einsele H & Knop S (2016). The Diagnosis and Treatment of Multiple Myeloma. *Dtsch Arztebl Int* **113**, 470-6.

2. Cid Ruzafa J, Merinopoulou E, Baggaley RF, Leighton P, Werther W, Felici D & Cox A (2016). Patient population with multiple myeloma and transitions across different lines of therapy in the USA: an epidemiologic model. *Pharmacoepidemiol Drug Saf* **25**, 871-9.

3. Kristinsson SY, Bjorkholm M, Andersson TM, Eloranta S, Dickman PW, Goldin LR, Blimark C, Mellqvist UH, Wahlin A, Turesson I & Landgren O (2009). Patterns of survival and causes of death following a diagnosis of monoclonal gammopathy of undetermined significance: a population-based study. *Haematologica* **94**, 1714-20.

4. Brigle K & Rogers B (2017). Pathobiology and Diagnosis of Multiple Myeloma. *Semin Oncol Nurs* **33**, 225-236.

5. Rajkumar SV (2018). Multiple myeloma: 2018 update on diagnosis, risk-stratification, and management. *Am J Hematol* **93**, 981-1114.

6. Landgren O, Kyle RA, Pfeiffer RM, Katzmann JA, Caporaso NE, Hayes RB, Dispenzieri A, Kumar S, Clark RJ, Baris D, Hoover R & Rajkumar SV (2009). Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. *Blood* **113**, 5412-7.

7. Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF & Melton LJ, 3rd (2002). A long-term study of prognosis in monoclonal gammopathy of undetermined significance. *N Engl J Med* **346**, 564-9.

8. Kyle RA, Durie BG, Rajkumar SV, Landgren O, Blade J, Merlini G, Kroger N, Einsele H, Vesole DH, Dimopoulos M, San Miguel J, Avet-Loiseau H, Hajek R, Chen WM, Anderson KC, Ludwig H, Sonneveld P, Pavlovsky S, Palumbo A, Richardson PG, Barlogie B, Greipp P, Vescio R, Turesson I, Westin J, Boccadoro M & International Myeloma Working G (2010). Monoclonal gammopathy of undetermined significance (MGUS) and smoldering

(asymptomatic) multiple myeloma: IMWG consensus perspectives risk factors for progression and guidelines for monitoring and management. *Leukemia* **24**, 1121-7.

9. Walker BA, Wardell CP, Melchor L, Brioli A, Johnson DC, Kaiser MF, Mirabella F, Lopez-Corral L, Humphray S, Murray L, Ross M, Bentley D, Gutierrez NC, Garcia-Sanz R, San Miguel J, Davies FE, Gonzalez D & Morgan GJ (2014). Intraclonal heterogeneity is a critical early event in the development of myeloma and precedes the development of clinical symptoms. *Leukemia* **28**, 384-390.

10. Dutta AK, Fink JL, Grady JP, Morgan GJ, Mullighan CG, To LB, Hewett DR & Zannettino ACW (2019). Subclonal evolution in disease progression from MGUS/SMM to multiple myeloma is characterised by clonal stability. *Leukemia* **33**, 457-468.

11. Das R, Strowig T, Verma R, Koduru S, Hafemann A, Hopf S, Kocoglu MH, Borsotti C, Zhang L, Branagan A, Eynon E, Manz MG, Flavell RA & Dhodapkar MV (2016). Microenvironment-dependent growth of preneoplastic and malignant plasma cells in humanized mice. *Nat Med* **22**, 1351-1357.

12. Gregersen H, Jensen P, Gislum M, Jorgensen B, Sorensen HT & Norgaard M (2006). Fracture risk in patients with monoclonal gammopathy of undetermined significance. *Br J Haematol* **135**, 62-7.

13. Kristinsson SY, Tang M, Pfeiffer RM, Bjorkholm M, Blimark C, Mellqvist UH, Wahlin A, Turesson I & Landgren O (2010). Monoclonal gammopathy of undetermined significance and risk of skeletal fractures: a population-based study. *Blood* **116**, 2651-5.

14. Golombick T & Diamond T (2008). Prevalence of monoclonal gammopathy of undetermined significance/myeloma in patients with acute osteoporotic vertebral fractures. *Acta Haematol* **120**, 87-90.

15. Ozaki A, Tsunoda M, Kinoshita S & Saura R (2000). Role of fracture hematoma and periosteum during fracture healing in rats: interaction of fracture hematoma and the periosteum in the initial step of the healing process. *J Orthop Sci* **5**, 64-70.

16. Hoff P, Gaber T, Strehl C, Schmidt-Bleek K, Lang A, Huscher D, Burmester GR, Schmidmaier G, Perka C, Duda GN & Buttgereit F (2016). Immunological characterization of the early human fracture hematoma. *Immunol Res* **64**, 1195-1206.

17. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC & Nauseef WM (2013). Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J Leukoc Biol* **93**, 185-98.

18. Klebanoff SJ (2005). Myeloperoxidase: friend and foe. *J Leukoc Biol* **77**, 598-625.

19. Hurst JK (2012). What really happens in the neutrophil phagosome? *Free Radic Biol Med* **53**, 508-20.

20. Guo W, Lu X, Liu Q, Zhang T, Li P, Qiao W & Deng M (2019). Prognostic value of neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio for breast cancer patients: An updated meta-analysis of 17079 individuals. *Cancer Med* **8**, 4135-4148.

21. Galdiero MR, Bonavita E, Barajon I, Garlanda C, Mantovani A & Jaillon S (2013). Tumor associated macrophages and neutrophils in cancer. *Immunobiology* **218**, 1402-10.

22. Panagopoulos V, Zinonos I, Leach DA, Hay SJ, Liapis V, Zysk A, Ingman WV, DeNichilo MO & Evdokiou A (2015). Uncovering a new role for peroxidase enzymes as drivers of angiogenesis. *Int J Biochem Cell Biol* **68**, 128-38.

23. Panagopoulos V, Leach DA, Zinonos I, Ponomarev V, Licari G, Liapis V, Ingman WV, Anderson P, DeNichilo MO & Evdokiou A (2017). Inflammatory peroxidases promote breast cancer progression in mice via regulation of the tumour microenvironment. *Int J Oncol* **50**, 1191-1200.

24. Ambrosone CB, Barlow WE, Reynolds W, Livingston RB, Yeh IT, Choi JY, Davis W, Rae JM, Tang L, Hutchins LR, Ravdin PM, Martino S, Osborne CK, Lyss AP, Hayes DF & Albain KS (2009). Myeloperoxidase genotypes and enhanced efficacy of chemotherapy for early-stage breast cancer in SWOG-8897. *J Clin Oncol* **27**, 4973-9.

25. Itonaga H, Imanishi D, Wong YF, Sato S, Ando K, Sawayama Y, Sasaki D, Tsuruda K, Hasegawa H, Imaizumi Y, Taguchi J, Tsushima H, Yoshida S, Fukushima T, Hata T, Moriuchi Y, Yanagihara K & Miyazaki Y (2014). Expression of myeloperoxidase in acute myeloid leukemia blasts mirrors the distinct DNA methylation pattern involving the downregulation of DNA methyltransferase DNMT3B. *Leukemia* **28**, 1459-66.

26. Nachbaur DM, Herold M, Maneschg A & Huber H (1991). Serum levels of interleukin-6 in multiple myeloma and other hematological disorders: correlation with disease activity and other prognostic parameters. *Ann Hematol* **62**, 54-8.

27. Vester H, Huber-Lang MS, Kida Q, Scola A, van Griensven M, Gebhard F, Nussler AK & Perl M (2014). The immune response after fracture trauma is different in old compared to young patients. *Immun Ageing* **11**, 20.

28. Treon SP & Anderson KC (1998). Interleukin-6 in multiple myeloma and related plasma cell dyscrasias. *Curr Opin Hematol* **5**, 42-8.

29. Dankbar B, Padro T, Leo R, Feldmann B, Kropff M, Mesters RM, Serve H, Berdel WE & Kienast J (2000). Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. *Blood* **95**, 2630-6.

30. Sezer O, Niemoller K, Eucker J, Jakob C, Kaufmann O, Zavrski I, Dietel M & Possinger K (2000). Bone marrow microvessel density is a prognostic factor for survival in patients with multiple myeloma. *Ann Hematol* **79**, 574-7.

31. Opperman KS, Vandyke K, Clark KC, Coulter EA, Hewett DR, Mrozik KM, Schwarz N, Evdokiou A, Croucher PI, Psaltis PJ, Noll JE & Zannettino AC (2019). Clodronate-Liposome Mediated Macrophage Depletion Abrogates Multiple Myeloma Tumor Establishment In Vivo. *Neoplasia* **21**, 777-787.

32. Friend NL, Hewett DR, Panagopoulos V, Noll JE, Vandyke K, Mrozik KM, Fitter S & Zannettino ACW (2020). Characterization of the role of Samsn1 loss in multiple myeloma development. *FASEB Bioadv* **2**, 554-572.

33.   Arthur A, Zannettino A, Panagopoulos R, Koblar SA, Sims NA, Stylianou C, Matsuo K & Gronthos S (2011). EphB/ephrin-B interactions mediate human MSC attachment, migration and osteochondral differentiation. *Bone* **48**, 533-42.

34.   DeNichilo MO, Panagopoulos V, Rayner TE, Borowicz RA, Greenwood JE & Evdokiou A (2015). Peroxidase enzymes regulate collagen extracellular matrix biosynthesis. *Am J Pathol* **185**, 1372-84.

35.   Friend N, Noll JE, Opperman KS, Clark KC, Mrozik KM, Vandyke K, Hewett DR & Zannettino ACW (2020). GLIPR1 expression is reduced in multiple myeloma but is not a tumour suppressor in mice. *PLoS One* **15**, e0228408.

36.   Panagopoulos V, Liapis V, Zinonos I, Hay S, Leach DA, Ingman W, DeNichilo MO, Atkins GJ, Findlay DM, Zannettino ACW & Evdokiou A (2017). Peroxidase enzymes inhibit osteoclast differentiation and bone resorption. *Mol Cell Endocrinol* **440**, 8-15.

37.   Vande Broek I, Vanderkerken K, Van Camp B & Van Riet I (2008). Extravasation and homing mechanisms in multiple myeloma. *Clin Exp Metastasis* **25**, 325-34.

38.   Manier S, Sacco A, Leleu X, Ghobrial IM & Roccaro AM (2012). Bone marrow microenvironment in multiple myeloma progression. *J Biomed Biotechnol* **2012**, 157496.

39.   Nakai Y & Nonomura N (2013). Inflammation and prostate carcinogenesis. *Int J Urol* **20**, 150-60.

40.   Allen MD & Jones LJ (2015). The role of inflammation in progression of breast cancer: Friend or foe? (Review). *Int J Oncol* **47**, 797-805.

41.   Thorsteinsdottir S, Gislason G, Aspelund T, Sverrisdottir I, Landgren O, Turesson I, Bjorkholm M & Kristinsson SY (2020). Fractures and survival in multiple myeloma: results from a population-based study. *Haematologica* **105**, 1067-1073.

42.   Ferrara N, Gerber HP & LeCouter J (2003). The biology of VEGF and its receptors. *Nat Med* **9**, 669-76.

43. Chen MF, Chen PT, Lu MS, Lin PY, Chen WC & Lee KD (2013). IL-6 expression predicts treatment response and outcome in squamous cell carcinoma of the esophagus. *Mol Cancer* **12**, 26.

44. Rosean TR, Tompkins VS, Tricot G, Holman CJ, Olivier AK, Zhan F & Janz S (2014). Preclinical validation of interleukin 6 as a therapeutic target in multiple myeloma. *Immunol Res* **59**, 188-202.

45. Kizaki M & Tabayashi T (2016). The Role of Intracellular Signaling Pathways in the Pathogenesis of Multiple Myeloma and Novel Therapeutic Approaches. *J Clin Exp Hematop* **56**, 20-7.

46. Fan L, Hong J, Huang H, Fu D, Wu S, Wang Q, Ye Y & Liu Y (2017). High Expression of Phosphorylated Extracellular Signal-Regulated Kinase (ERK1/2) is Associated with Poor Prognosis in Newly Diagnosed Patients with Multiple Myeloma. *Med Sci Monit* **23**, 2636-2643.

47. Jung SH, Ahn SY, Choi HW, Shin MG, Lee SS, Yang DH, Ahn JS, Kim YK, Kim HJ & Lee JJ (2017). STAT3 expression is associated with poor survival in non-elderly adult patients with newly diagnosed multiple myeloma. *Blood Res* **52**, 293-299.

48. Rymaszewski AL, Tate E, Yimbesalu JP, Gelman AE, Jarzembowski JA, Zhang H, Pritchard KA, Jr. & Vikis HG (2014). The role of neutrophil myeloperoxidase in models of lung tumor development. *Cancers (Basel)* **6**, 1111-27.