Role of Wnt/β-catenin and CXCL12/CXCR4 signalling axes in the damage and recovery of the bone marrow microenvironment following methotrexate chemotherapy

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Declaration

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Acknowledgements

Abstract

The bone marrow microenvironment is home to mesenchymal and haematopoietic stem cells and their respective progeny. Mesenchymal stem cells are multipotent and have the capacity to differentiate into a number of cell types, namely osteoblasts, adipocytes and chondrocytes. These cells and cells of the haematopoietic lineage maintain close interactions within the marrow cavity and are responsible for bone and bone marrow maintenance throughout life. Disruptions to cell populations and steady-state interactions within the bone marrow such as that seen following cancer chemotherapy treatment are associated with bone-related complications in later life such as osteoporosis. However, the underlying mechanisms of these defects and the subsequent recovery potential remain unclear. The studies presented herein have investigated the effects of the commonly used antimetabolite methotrexate (MTX) on the damage and recovery of the bone marrow microenvironment and potential signalling pathways involved, focusing on Wnt/β-catenin and CXCL12/CXCR4 signalling axes. Using a short-term rat MTX model of 5 consecutive daily doses at 0.75mg/kg, histological techniques were employed to assess bone/fat formation and cell culture techniques were used to investigate differentiation potential of bone marrow mesenchymal and haematopoietic cells. These investigations were further supported by protein expression and quantitative RT-PCR analyses of associated genes over the MTX time-course.

The bone marrow cavity was observed to undergo a number of changes when assessed histologically, with damage obvious on days 6 and 9 and recovery apparent by day 14. This was identified by an increased adipogenic marrow and reduced trabecular bone volume, parallel to a reduction in mineralising potential yet increased adipogenic potential of isolated marrow stromal cells. This was further supported by changes in bone marrow stromal cell gene expression, whereby adipogenic transcription factor PPARγ was increased concurrent to a reduction in osteogenic transcription factor Osterix, indicating a switch in lineage commitment. In order to characterise molecular mechanisms

underlying such altered lineage commitment, the role of Wnt/β-catenin signalling was investigated, known to critically function in mesenchymal stem cell differentiation. Interestingly, MTX induced notable changes in Wnt signalling-associated genes assessed in the stromal cell population. Concurrent administration of the synthetic GSK-3β inhibitor BIO abrogated the above transient changes in bone/fat volumes, osteogenic/adipogenic commitment and gene expression. This demonstrates a potential role for Wnt/β-catenin signalling in MTX chemotherapy-induced changes to osteogenic/adipogenic commitment and a therapeutic potential for preventing bone loss and marrow adiposity by promoting Wnt signalling via GSK-3β inhibition.

Furthermore, to clarify the mechanisms associated with the recovery response of the bone marrow microenvironment, the current project also examined the CXCL12/CXCR4 signalling axis, known to be involved in mobilisation, homing and maintenance of a quiescent stem cell pool, enabling reestablishment of a functioning marrow in response to damaging conditions. After MTX, coinciding with the reduction of marrow cellularity, CXCL12 protein expression was observed to decrease on day 9, accompanied by an increase in CXCL12-degrading metalloproteinase MMP-9. *In vitro* studies confirmed that recombinant MMP-9 was able to degrade CXCL12 protein. In addition, changes in gene expression of CXCL12 and its receptor CXCR4 in the bone marrow stromal cell population as well as the non-adherent fraction were observed following MTX treatment. This further suggests the CXCL12/CXCR4 axis is deregulated over the MTX damage/repair time-course and is potentially involved in the regulation of bone marrow damage and recovery.

Chapter 1

Damage and Recovery of the Bone Marrow Microenvironment Induced by Cancer Chemotherapy – Potential Regulatory Chemokine CXCL12/Receptor CXCR4 Signalling

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Role of the Wnt/β-catenin signalling pathway in bone formation

Wnt signalling was first described in *Drosophila* and is a highly conserved pathway, whose name comes from the polarity gene wingless in *Drosophila* [1]. Wnt molecules exert their effects through canonical and non-canonical pathways, with the canonical or β-catenin-dependent pathway more clearly defined. The canonical Wnt/β-catenin signalling pathway involves a number of ligands, receptors and antagonists that function to regulate cell commitment, differentiation and proliferation [2]. Secreted Wnt ligands bind to the frizzled (Fzd) and co-receptor lipoprotein-related 5/6 (Lrp5/6) complex, whereby activation of Fzd recruits the cyotoplasmic molecule dishevelled (Dsh), causing inhibition of glycogen synthase kinase-3β (GSK-3β), which in turn stabilises β-catenin. (Figure 1). Stabilisation and accumulation of β-catenin allows its translocation to the nucleus and in turn initiates activation of T-cell factor/lymphoid enhancing factor (TCF/LEF)-mediated transcription of target genes eliciting a variety of effects including proliferation and differentiation. Furthermore, several secreted molecules are antagonists of Wnt signalling, including secreted frizzled-related proteins (sFRPs) and secreted glycoproteins dickkopfs (Dkks) [3-4]. The significance of this complex signalling pathway in the clinic has been highlighted by increased bone mass due to LRP5 mutations that prevent Dkk-1 binding [5]. It has been established that Wnt signalling is critical for bone formation and maintenance and that canonical signalling promotes stromal progenitor proliferation and osteoblastogenesis, whilst inhibiting adipogenesis [6-7]. Under steady-state conditions, the Wnt/β-catenin signalling pathway regulates commitment of mesenchymal stem cells to the osteogenic lineage, maintaining a balance between osteoblast and adipocyte differentiation. Recent investigations have illustrated the integral role of Wnt ligand Wnt10b, in maintaining pre-adipocytes in an undifferentiated state [8-9] further illustrating the role the Wnt/β-catentin signalling pathway in regulation of a steady-state functioning bone marrow microenvironment.

Under conditions of stress or injury, such as those induced by ovariectomy, glucocorticoid treatment, aging and chemotherapy, inducing defects such as bone loss and myelosuppression, a fatty marrow phenotype is also observed, characteristic of osteoporosis [10-15]. In this case, there is an increase in adipogenic differentiation potential of MSC within the marrow cavity at the expense of osteogenic differentiation. Due to its importance in the commitment and differentiation of MSC within the bone marrow cavity, the Wnt/β-catenin signalling pathway was postulated to be involved in the deregulated lineage commitment and subsequent differentiation of damaged marrow cell populations.

Under steady-state conditions, bone formation is not maintained solely by regulatory pathways controlling MSC commitment and differentiation such as the Wnt/β-catenin signalling pathway. Osteoclasts, derived from haematopoietic cells, act in conjunction with osteoblasts to maintain optimal bone mass throughout life. Osteoblasts and osteoclasts have a mutually dependent relationship, whereby osteoclasts degrade bone and osteoblasts form new bone. It has been previously illustrated that several Wnt ligands regulate monocyte expansion from haematopoietic progenitor cells within the bone marrow microenvironment, demonstrating a role, however unclear, of the Wnt signalling pathway in regulation of osteoclastogenesis [16-17]. In multiple myeloma patients, increased osteoclast activity was reduced with Wnt3a over-expression or inhibition of the Wnt signalling antagonist Dkk-1 [18]. These studies illustrate the essential role the Wnt/β-catenin signalling pathway plays on cell populations of the bone marrow microenvironment, allowing their subsequent interactions in order to regulate bone formation and degradation throughout a life-time.

Glycogen synthase kinase 3β (GSK-3β) is a cytosolic inhibitor of Wnt signalling, acting with the degradation complex of Axin, APC and Ck1α to phosphorylate β-catenin, targeting it for ubiquitin-

mediated degradation by the proteosome. Phosphorylation of serine/threonine residues in GSK-3β have been illustrated to inhibit GSK-3β activity [19]. It was originally named for its involvement in glycogen metabolism and regulation of metabolic enzymes such as glycogen synthase [19-20] and exists in 2 isoforms, α and β, 51-kDa and 47-kDa respectively, sharing 95% conformity in the kinase domain. Interestingly, GSK-3β loss-of-function mutations result in stabilisation of β-catenin protein and constitutive activation of Wnt signalling, allowing the possibility of potential therapeutic applications of GSK-3β inhibition [21].

The synthetic ATP-competitive GSK-3β inhibitor, 6-bromoindirubin-3'-oxime, BIO, has previously been identified as having the capacity to modulate mesenchymal differentiation potential down both osteogenic and adipogenic lineages [22-23]. Interestingly pre-treatment of hMSCs with BIO under osteogenic conditions caused increased mineralisation in a dose-dependent manner [22]. Furthermore, BIO has been illustrated to abrogate glucocorticoid-induced reduction in bone mass and osteogenic differentiation potential, as well as dampening the increased adipogenic potential observed following glucocorticoid treatment in a rat model [23]. As the Wnt signalling pathway is associated with commitment and differentiation potential of mesenchymal precursors, Wnt-associated genes and the application of the GSK-3β inhibitor BIO, have been investigated in the following chapters and will be discussed below.
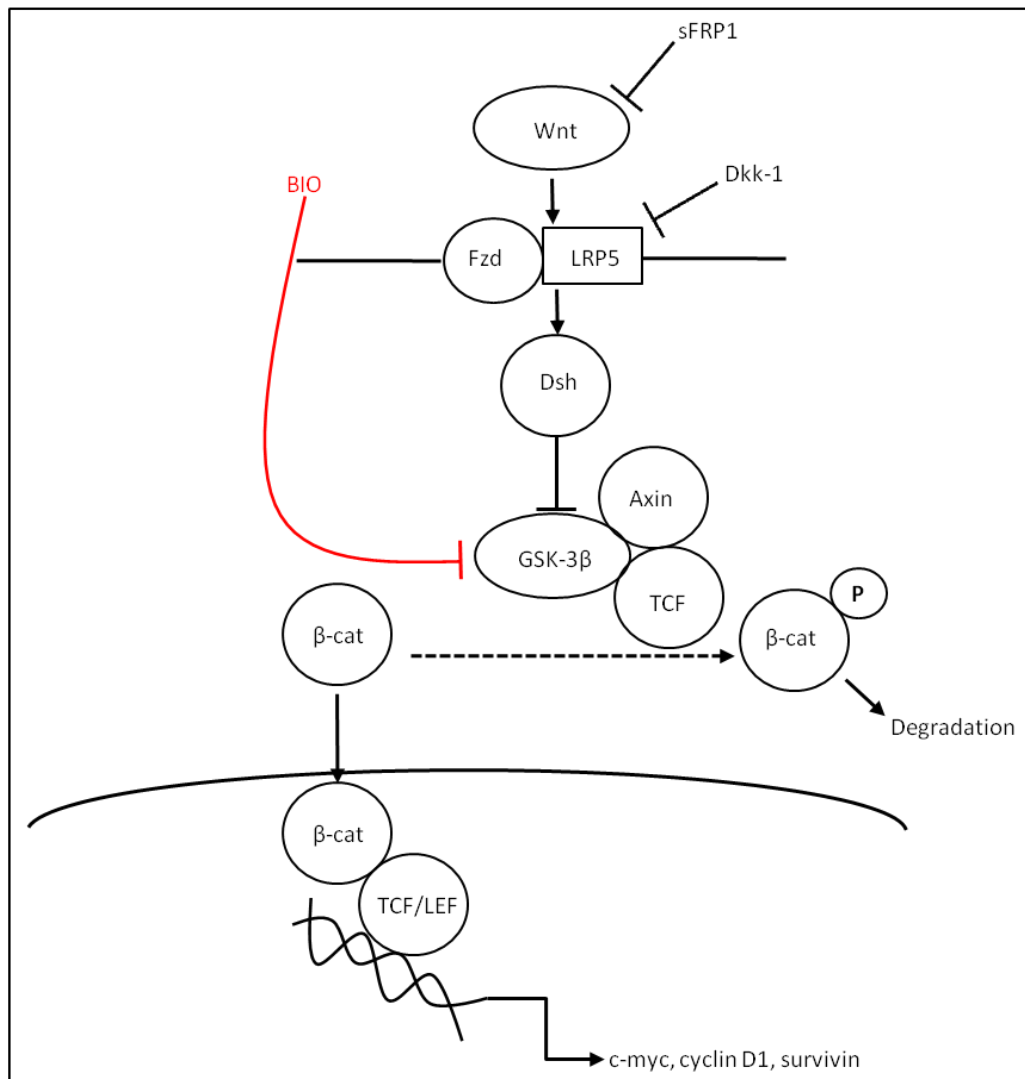
Figure 1: Wnt/B-catenin signalling and the inhibitory action of 6-bromoindirubin-3'-oxime (BIO).

Research Summary

The scope of this research project has been to identify the effects of methotrexate (MTX) chemotherapy on bone marrow cell populations and their subsequent recovery potential, and to investigate potential signalling mechanisms associated with the effects observed over the treatment time-course in an attempt to enhance our understanding of chemotherapy-associated bone defects.

Hypothesis

The increased marrow adiposity and reduced bone volume observed following methotrexate treatment is induced by deregulated Wnt signalling resulting in altered mesenchymal stem cell commitment in favour of the adipogenic lineage at the expense of osteogenesis. Furthermore, the damage and recovery of the bone marrow microenvironment observed as changes to stem and progenitor cell populations of the haematopoietic lineage is associated with methotrexate-induced deregulation of the CXCL12/CXCR4 axis.

Aims

The aims of this project are contained in the studies outlined below.

Study 1: To study the pathophysiology of MTX-induced bone loss, Study 1 examined effects on bone and marrow fat volume, population size and differentiation potential of bone marrow stromal cells (BMSC) in adult rats following chemotherapy over a short-term.

Study 2: Since the Wnt/β-catenin signalling pathway has been identified as integral in regulating bone formation and limiting adipogenesis, the current study investigated the potential role of Wnt/β-catenin signalling in osteogenic and adipogenic commitment of bone marrow stromal cells after MTX chemotherapy. In addition, the current study examined the therapeutic potential of modulating Wnt signalling for the prevention of bone loss and marrow adiposity through the

inhibition of glycogen synthase kinase 3β (GSK-3β), a component of the complex involved in destabilising β-catenin.

Study 3: Since the underlying mechanisms and recovery potential of chemotherapy-induced myelosuppression and bone loss remain unclear and the CXCL12/CXCR4 chemotactic axis has been demonstrated to be critical in progenitor cell homing to regions of need upon injury, herein the rat model of acute MTX chemotherapy was used to investigate potential involvement or deregulation of CXCL12/CXCR4 axis in damage and recovery of the bone marrow cell pool.

Chapter 2

Methotrexate chemotherapy reduces osteogenesis but increases adipogenic potential in the bone marrow

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MA Scherer

Carried out animal trials and specimen collection in conjunction with KR Georgiou. Initial conceptualisation of study and experiments carried out for and associated with Figure 4B-D performed by MA Scherer.

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Chapter 3

Methotrexate chemotherapy-induced changes to osteogenesis and adipogenesis are associated with the Wnt signalling pathway

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Chapter 3

Statement of Authorship

Methotrexate chemotherapy-induced changes to osteogenesis, adipogenesis and haematopoiesis are associated with the Wnt signalling pathway

Journal of Clinical Investigation 2010: submitted paper

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Chapter 4

Deregulation of the CXCL12/CXCR4 axis in methotrexate chemotherapy-induced damage and recovery of the bone marrow microenvironment

Kristen R Georgiou[1, 2], Michaela A Scherer[1, 3],Tristan J King[1, 2], Bruce K Foster[3] and Cory J Xian[1, 2, 3, 4]

Statement of Authorship

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Deregulation of the CXCL12/CXCR4 axis in methotrexate chemotherapy-induced damage and recovery of the bone marrow microenvironment

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Key Words:

- **Methotrexate chemotherapy**

- **CXCL12**

- **CXCR4**

- **Haematopoietic cells**

- **Stromal cells**

- **Bone marrow**

Tables: 1, **Figures:** 5.

Abstract

Cancer chemotherapy disrupts bone marrow microenvironment affecting steady-state proliferation, differentiation and maintenance of haematopoietic and stromal stem and progenitor cells. However, the underlying mechanisms and recovery potential of chemotherapy-induced myelosuppression and bone loss remain unclear. The CXCL12/CXCR4 chemotactic axis has been demonstrated to be critical in maintaining interactions between cells of the two lineages and progenitor cell homing to regions of need upon injury. Here a rat model of chemotherapy with the commonly used antimetabolite methotrexate (MTX) (five once-daily injections at 0.75mg/kg/day) was used to investigate potential roles of CXCL12/CXCR4 axis in damage and recovery of the bone marrow cell pool. After MTX treatment, reduced marrow cellularity was found, accompanied by altered CXCL12 protein levels (increased in peripheral blood but decreased in bone marrow) and reduced CXCR4 mRNA expression in bone marrow cells. Accompanying the lower marrow CXCL12 protein levels (despite its increased mRNA expression in stromal cells) were the increased gene and protein levels of metalloproteinase MMP-9 in bone and bone marrow and lack of changes in expression of MMP-9 inhibitor TIMP-1. Furthermore, recombinant MMP-9 was able to degrade CXCL12 *in vitro*. In addition, while there was an increase in colony formation of granulocyte and macrophage progenitor cell lineage (CFU-GM) after MTX treatment, exogenous CXCL12 was not found to affect CFU-GM potential of normal marrow cells. These findings suggest that MTX chemotherapy transiently alters bone marrow cellularity and composition and that the reduced cellularity may be associated with increased MMP-9 expression and deregulated CXCL12/CXCR4 chemotactic signalling.

Introduction

The bone marrow microenvironment is home to two distinct stem cell types, haematopoietic (HSCs) and mesenchymal stem cells (MSCs). Maintenance of an optimally functioning bone marrow and as a result, haematopoiesis, bone formation and remodelling is largely dependent upon interactions between these two cell types and their progeny. The bone marrow is the site of mesenchymal stem cell commitment and differentiation as well as the site of haematopoiesis and regulated release of haematopoietic cells into the circulation throughout a lifetime (1-2).

Stem cells are housed at endosteal niche sites, which are a supportive environment maintaining HSCs in a quiescent state via tight adhesive interactions between bone-lining osteoblasts and HSCs involving molecular interactions such as VCAM/VLA-4, CXCL12/CXCR4, SCF/c-Kit, Jagged/Notch and Ang-1/Tie-2 (3-6). The importance of the chemotactic CXCL12/CXCR4 axis in homing and retention of stem cells has become more defined, as demonstrated by *in utero* death of CXCL12 or CXCR4 knock-out mice due to major developmental defects, including haematopoiesis (7-8). Chemokine CXCL12 (also known as stromal derived factor 1, SDF-1) is expressed by osteoblasts, bone marrow stromal cells, endothelial and perivascular cells and is well-established as a chemoattractant. CXCL12 binds to its G-protein coupled receptor CXCR4, expressed on haematopoietic stem and progenitor cells and a portion of stromal stem or mesenchymal stem cells, clearly illustrating the interaction between the two cell lineages (9). CXCL12 and CXCR4 were thought to be a monogamous pair until recent times, when an orphan receptor CXCR7 was identified to bind CXCL12 with a strong affinity (10). While CXCR7 deficiency also resulted in lethality, there were no disruptions to the foetal haematopoietic system. Therefore, the importance of the CXCL12/CXCR4 interaction in homing, mobilisation and establishment of the bone marrow microenvironment remains clear (10-12).

Under steady-state conditions the number of circulating haematopoietic progenitors is low; however under conditions of stress or injury when mobilisation occurs, these numbers are found to increase (13). Medical treatments such as myeloablative therapies including chemotherapy regimens induce cell cycling of quiescent stem cells and their mobilisation out of the bone marrow, increasing circulating haematopoietic progenitors (5, 14). Such procedures are used in the clinic in order to obtain an optimal number of haematopoietic stem and progenitor cells for autologous stem cell transplantation following cancer chemotherapy treatment to re-establish a depleted bone marrow microenvironment (15). Stem cell mobilisation may be a result of disruptions to the above mentioned molecular interactions releasing HSCs into more central marrow regions and inducing their cycling. Thus long-term ablative therapies reduce the HSC pool available to re-establish the continually depleted marrow cavity. This in turn may contribute to deregulation of bone remodelling and turnover, resulting in overall bone loss (12).

It has been suggested that several possibilities involving the CXCL12/CXCR4 axis may contribute solely or in combination to allow this to occur. Firstly, accumulation of neutrophils results in an increased release of neutrophil elastase (NE) and cathepsin G (16), which have been shown to cleave both CXCL12 and CXCR4, disrupting their adhesive interaction (17). As a result, allowing HSCs held in a quiescent state at the endosteal niche to move into the cell cycle. This remains controversial however, as mobilisation in NE x CG deficient mice remained normal (18), indicating a NE and CG-independent mechanism of posttranslational CXCL12 or CXCR4 cleavage (18). Secondly, associated with increased osteoclast activity under stress conditions is an increase in matrix degrading enzyme matrix metalloproteinase (MMP-9, gelatinase B) and the potent bone resorbing protease cathepsin K, which have been associated with a reduced

CXCL12 protein concentration (18-21). Consistently, MMP-9 has been illustrated to directly cleave CXCL12 at its N-terminus (22). Thirdly, another hypothesis of axis deregulation is that CXCL12 and/or CXCR4 transcription is reduced in resident cells or direct blockage of the CXCL12/CXCR4 interaction occurs (23-24). Alternatively a gradient change in CXCL12 protein level in favour of the peripheral blood (25) over the bone marrow (BM) has also been postulated as a possible cause of the observed migration out of the BM (22, 26). However, the exact mechanism enabling mobilisation/recovery to occur remains largely elusive and needs to be further studied.

Side effects of chemotherapy treatments are varied in their severity and targets depending on the dosage and agent(s) used. Bone marrow myelosuppression, indicated by reduced haematopoietic cellularity and differentiation potential both in *in vitro* and *in vivo* studies has been demonstrated to be a side-effect associated with cancer chemotherapy (27-30). Similarly, steady-state maintenance of the stromal lineage is also disrupted, with high-dose chemotherapy resulting in a depleted osteogenic precursor population of the bone marrow of adult cancer patients (31), reducing osteogenic differentiation potential and bone formation (32-34). Consequently, improved cancer patient survival rates have been associated with the increased observations of long-term skeletal side effects, where chemotherapy is commonly associated with bone defects such as osteoporosis and increased fracture risk in paediatric and adult cancer patients and survivors (35-38). However, despite these findings, the mechanisms by which chemotherapy causes bone marrow stromal progenitor cell damage and bone defects are yet to be elucidated and it remains to be investigated whether the CXCL12/CXCR4 interaction axis is associated with the bone marrow stromal progenitor cell damage and recovery following chemotherapy.

Methotrexate (MTX), is a commonly used anti-metabolite that inhibits dihydrofolate reductase, lowering cellular pools of folate and inhibiting purine and pyrimidine synthesis (39-40). MTX is typically used in consolidation and maintenance therapies for acute lymphoblastic leukaemia and adult cancers and its use is associated with myelosuppression, bone loss in paediatric and adult cancer patients and in experimental animals. We have shown recently that acute MTX treatment causes decreased bone formation and osteoporosis-like effects in rat models (41-42). In addition, acute treatment with MTX can cause a transient reduction in the proliferation of bone marrow stromal progenitor cells (42). However the extent to which MTX damages the bone marrow microenvironment and the degree it recovers remain largely unknown. Furthermore, whether deregulation or regulation of CXCL12/CXCR4 axis is involved in MTX chemotherapy-induced bone marrow damage and regeneration remains to be studied. In the current study, a rat model of chemotherapy with MTX was used to investigate the damage and regeneration of the bone marrow haematopoietic and stromal cell populations and the potential roles and deregulation of the CXCL12/CXCR4 axis in these events.

Materials and Methods

Animal trials and Tissue Collection and Processing. Sprague Dawley rats of approximately 150g in body weight were used for a short-term trial receiving subcutaneous MTX administration at a therapeutic dose of 0.75mg/kg for 5 consecutive days as described (41, 43). Rats were euthanized at days 6, 9, 10, 12, 14, or 21 after the first dosing (n = 7 to 9 rats/group) in order to assess the time course of injury and recovery of the bone marrow microenvironment. A group of saline-injected rats were used as normal controls. The protocol followed the Australian Code of Practice for the Care and Use of Animals and was approved by the Animal Ethics Committee of

the Women's and Children's Hospital, Institute of Medical and Veterinary Science, and University of South Australia.

After euthanasia, a cardiac puncture was immediately performed to obtain peripheral blood in lithium-heparin collection tubes for obtaining plasma samples, after which tibiae, femurs and pelvises were dissected. To obtain bone marrow cells for cell culture studies, the left tibia and left femur were flushed (see below). For histology and immunohistochemical studies, the right tibial specimens were fixed in 10% formalin overnight and decalcified in Immuocal (Decal Corporation, Tallman, NY) solution for 14 days at 4°C prior to being bisected longitudinally with one half processed routinely and embedded in paraffin wax for producing 4µm sections which will be stained by H&E as previously described (44). The metaphyseal region of the right femur was collected, snap frozen and stored at -80°C for RNA extraction and gene expression studies.

Bone marrow cell isolation and cell culture. Bone marrow samples obtained from the same animal were combined, resuspended in 2ml of basal media and passed through a 19-gauge needle, followed by a 70µm nylon filter cell strainer (BD Biosciences, NSW, Australia) for removal of contaminating particles. The suspension was diluted by addition of an equal volume of PBS and then overlaid on 4ml of Lymphoprep™ density gradient and centrifuged at a speed of 800g for 20 minutes with the brake set to 0. The bone marrow mononuclear cell (BMMNC)-containing interface was collected, washed with PBS and pelleted cells were resuspended in basal media for routine dye exclusion cell viability and density counts using Trypan blue (Sigma-Aldrich, NSW, Australia). In order to obtain bone marrow stromal cells (BMSC) for further culture, 200µl of the bone marrow suspension was plated into T25 flasks and 1ml into a T75 flask, maintained at 37°C and 5% CO_2 until 80% confluence was achieved (typically after 10 days).

CFU-GM Assay. As a means to assess the effects of MTX treatment on changes to haematopoietic lineage differentiation, colony forming unit-granulocyte macrophage (CFU-GM) assay was performed with bone marrow cell specimens. Non-plastic adhering cells of the haematopoietic lineage were plated out in MethoCult, a semi-solid methylcellulose gel containing cytokines (Stem Cell Technologies, VIC, Australia). Bone marrow mononuclear cells were plated out at 1.5×10^4 in 0.3ml of MethoCult per well in a 24-well plate, with added stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3), which are the minimum requirements for colony formation and expansion based on previous studies (45). After 14 days of culture at 37°C and 5% CO_2, cell growth was assessed and aggregates of 50 or more cells were counted as colonies. In order to assess the role of CXCL12 in the proliferation of both control specimens or MTX-treated haematopoietic progenitors, 50µg/ml recombinant CXCL12 (R and D Systems) was added to the MethoCult medium on the day of seeding together with or without 100nM MTX (46). CFU-GM colonies were assessed on day 11 of culture as colonies/1.5×10^4 MNCs.

CFU-F assay. In order to assess the bone marrow stromal population response to exogenous CXCL12 and MTX *in vitro*, isolated stromal cells were used to set up CFU-fibroblast (CFU-F) assay as described (47). Briefly, 100nM MTX or control was added to the basal medium of cultured BMSCs 24 hours after seeding and 50µg/ml recombinant CXCL12 or control was added a further 24 hours later. CFU-F colonies counted as aggregates of 50 or more cells, were assessed after 14 days of culture and stained for both alkaline phosphatase (representing osteoprogenitor cell differentiation potential) and toluidine blue (representing total progenitor cell differentiation).

RNA Isolation and RT-PCR Gene Expression Analysis. <u>RNA Isolation:</u> In order to assess gene expression of bone marrow cells after MTX treatment in rats, isolated primary stromal cells were grown in a T75 flask with basal medium until confluence, at which time the cells were collected and frozen at -80°C until time of RNA extraction. RNA isolation of BMSC pellet was performed using RNAqueous®-Micro Kit (Ambion, Applied Biosystems Pty Ltd, VIC, Australia) following standard protocol and RNA was further purified of contaminating DNA. For metaphyseal bone gene expression studies, frozen total metaphyseal bone specimens were ground to fine powder using a mortar and pestle and liquid nitrogen. Total RNA was then extracted using TRI reagent (Sigma, Australia). For gene expression studies of blood cells in peripheral blood, 1ml whole blood was collected with a syringe containing EDTA and 500µl was stored in a 2ml tube pre-loaded with RNA-*later* at -80°C. Isolation was performed using Mouse RiboPure™-Blood RNA Isolation Kit (Ambion) which is also suitable for rat whole blood and whole bone marrow RNA extraction and the resulted RNA was purified of contaminating DNA using TURBO DNase-*free* Kit (Ambion).

<u>Real- time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):</u> First strand cDNA was reverse transcribed at a total 2µl reaction, containing 1µl random decamers (50ng/µl, GeneWorks, SA, Australia), 1µl 10mM dNTP mix and 2µg total RNA and Superscript™ III Reverse Transcriptase as instructed (Invitrogen Pty Ltd, VIC, Australia). cDNA was also synthesised without the addition of reverse transcriptase as a negative control. cDNA templates were used for amplification by PCR. All primers (Table 1) were designed using PRIMER BLAST to ensure forward and reverse primers in different exons and were obtained from GeneWorks. Design parameters were set at: 80-150bp in length, CG content 50-60% and Tm2 58-62. For RT-PCR of genes of interest, SYBER green® PCR reactions with the cDNA samples were run in

parallel with internal control gene Cyclophilin A on a 7500 Fast Real-Time PCR System (Applied Biosystems) as described (42, 47-48). Relative expression against Cyclophilin A was calculated using the comparative Ct ($2^{-\Delta Ct}$) method.

Table 1: Primer pairs used in this study

Gene of interest	Forward	Reverse
CXCL12	ATCAGTGACGGTAAGCCAGTCA	TGCACACTTGTCTGTTGTTGCT
CXCR4	ATGTGAGTTCGAGAGCGTCGT	TGGAATTGAGTGCATGCTGC
MMP-9	TCGAAGGCGACCTCAAGTG	GCGGCAAGTCTTCGGTGTAG
Cyclophilin A	GAGCTGTTTGCAGACAAAGTTC	CCCTGGCACATGAATCCTG

Enzyme Linked Immunosorbent Assays (ELISA) for CXCL12, MMP-9 and TIMP-1. Protein concentrations of CXCL12, MMP-9, and TIMP-1 in the bone marrow supernatant and/or blood plasma were determined by ELISA in specimens from rats treated with or without MTX. Bone marrow aspiration from individual rat pelvises with 250ul of PBS was performed to collect the supernatant fraction of the bone marrow. Similarly, peripheral blood plasma was collected from each individual rat and stored at -20°C. For ELISA, samples were diluted 1:4 and standards were prepared with recombinant mouse CXCL12/SDF-1α, human MMP-9 or rat TIMP-1 (460-SD, 911-MP, 580-RT, R&D Systems) in diluent with ranges of concentrations beginning from 5ng/ml for CXCL12 and 2ng/ml for MMP-9 and TIMP-1. Wells were coated with monoclonal anti-human/mouse CXCL12/SDF-1 antibody, anti-human MMP-9 antibody or anti-rat TIMP-1 antibody (MAB350, MAB936, MAB5802, R&D Systems) and stored at -20°C overnight. After washes, wells were blocked with 1% BSA/PBS for 2 hours, washed and added with 100µl of the standards and samples (1:4). After being incubated for a further 2 hours and washed, a biotinylated anti-human/mouse CXCL12/SDF-1 antibody, anti-human MMP-9 antibody or anti-rat TIMP-1 antibody

(BAF310, BAF911, BAF580, R&D Systems) was added and incubated for 2 hours. After wash, streptavidin-HRP (DY998, R&D Systems) and tetramethylbenzidine (TMB) liquid substrate (P7998, Sigma-Aldrich) were used for colour development. After adding 1M H_2SO_4 stop solution, optical density (OD) was measured immediately using a microplate reader set to 450nm and wavelength correction was set to 570nm. Once an optimised linear standard curve was achieved, sample concentrations were analysed in triplicate with a corresponding standard curve for comparison and graphed in ng/ml.

CXCL12 Western Blotting. In order to assess the potential proteolytic effect of MMP-9 on CXCL12 protein, CXCL12 western blotting was employed to analyse the *in vitro* incubation reaction products of recombinant MMP-9 on CXCL12. Recombinant CXCL12 (50ng) (R&D systems) was incubated with or without recombinant MMP-9 protein (100ng) (911-MP, R&D Systems) for 8 hours at 37°C (22) and the reaction was terminated by addition of SDS-polyacrilamide gel electrophoresis (49) loading buffer. Samples were run on a 15% polyacrylamide gel alongside with a pre-stained protein ladder (10748010 BenchMark™, Invitrogen Pty Ltd, VIC, Australia), transferred onto a nitrocellulose membrane (Pall Life Sciences, VIC, Australia) using the semi-dry method (Bio-Rad Laboratories Pty Ltd, NSW, Australia). After blocking with 5% non-fat milk powder for 2 hours at 4°C, membranes were incubated with 2μg/ml of anti-human/mouse CXCL12/SDF-1 antibody (MAB350, R&D Systems) overnight at 4°C. Membranes were then incubated with biotinylated rabbit anti-mouse secondary antibody (Dako, NSW, Australia) (1:600) for one hour and followed by strepdavidin-HRP (R&D systems) (1:200) for 30 minutes. Enzyme chemiluminescence (ECL) was read subsequent to incubation of the membrane with appropriate substrate solution.

Statistical Analyses. Standard One-way ANOVA with Tukey post test was performed using GraphPad Prism (5.01 for Windows, GraphPad Software, San Diego CA, USA). Significance was achieved when $p < 0.05$, where different superscript letters in figures denote mean values being significantly different from each other.

Results

Changes in bone marrow cell density and haematopoietic precursors. Consistent with the obvious reduction in bone marrow cellularity particularly on day 9 after the first MTX dosing (Figure 1A, 1B), viable cell densities of bone marrow aspirates as assessed by trypan blue dye exclusion were found to be significantly decreased on day 6 (6.06×10^6 cells/ml), day 9 (18.26×10^6 cells/ml) and day 10 (22.01×10^6 cells/ml), when compared to control (45.89×10^6 cells/ml). By day 14, the viable cell density returned close to control (36.24×10^6 cells/ml) (Figure 1C).

Despite evidence of reduced haematopoietic cellularity on day 9 after MTX treatment, a CFU-GM assay of bone marrow aspirates indicated that on day 6 there was an increase in colony formation of granulocyte and macrophage progenitor cell lineage, although CFU-GM formation at all other time-points returned to normal or appeared unchanged (Figure 1D). This suggests that among the unaffected cells in the bone marrow on day 6, a greater portion of these have the capacity to differentiate along the granulocyte/macrophage lineage potentially to enrich the pools of haematopoietic precursors for achieving bone marrow recovery.

Reduced CXCR4 expression correlates to depleted BM cellularity. Since previous studies have established the notion that antagonising CXCR4 or posttranslational cleavage of CXCR4 results in mobilisation of progenitor cells to the circulation (5), in the current study we assessed the mRNA expression of CXCR4 in BM non-adherent haematopoietic cells and in cells of peripheral blood. There was an observed reduction in CXCR4 gene expression in both the whole BM specimens on day 9 (Figure 2A) and the BM non-adherent cell fraction on day 6 and day 9 (Figure 2B), correlating with the periods of reduced bone marrow cellularity. This suggests that indeed deregulation of CXCL12/CXCR4 signalling is occurring in this MTX chemotherapy-induced stress, potentially associated with the reduced haematopoietic cell density observed in the BM cavity. However, CXCR4 mRNA expression remained relatively unchanged in blood specimens on all time points (Figure 2C).

Changes in CXCL12 expression associated with reduced BM cellularity. Since the well characterised chemotactic function of CXCL12 (mediated by the G-protein coupled receptor CXCR4) is potentially involved in G-CSF, stress and chemotherapy-induced progenitor cell mobilisation, we investigated effects of MTX treatment on BM and PB plasma CXCL12 protein levels. Following MTX treatment, CXCL12 protein expression in the bone marrow supernatant was found to be reduced significantly on day 9 and returned to control levels by day 14 (Figure 3A). In contrast, CXCL12 protein expression in the blood plasma was found to increase significantly on day 6 when compared to control samples and remained unchanged at other time-points following MTX treatment (Figure 3B).

To further investigate the source of MTX-induced changes to CXCL12 regulation, we assessed CXCL12 mRNA expression in cultured BMSCs from MTX-treated rats. On day 9, CXCL12 gene expression was found to be significantly greater than control levels and declined again by day 14 (Figure 3C), suggesting BMSCs may up-regulate their expression of CXCL12 in order to allow progenitor cells homing back to the bone marrow microenvironment in order to re-establish the depleted BM cavity.

Increased MMP-9 expression associated with reduced CXCL12 protein concentrations. Previous studies have indicated that MMP-9 degrades CXCL12, disrupting the CXCL12/CXCR4 interaction when assessed both *in vitro* and *in vivo* under conditions of G-CSF-induced haematopoietic progenitor mobilisation (17, 22, 50). In view of the reduction in CXCL12 protein expression yet an increase in gene expression on day 9 following MTX-induced damage, we hypothesised that this could be due to degradation of CXCL12 protein by MMP-9. As an initial step to investigate this possibility, metaphyseal bone was assessed for MMP-9 mRNA expression on day 6, day 9 and day 14 after MTX treatment as compared to normal control. On day 6, MMP-9 gene expression was found to be significantly increased which then declined slightly on day 9 and back to control levels by day 14 (Figure 4A). Consistent with the increased CXCL12 protein level in the blood plasma on day 6 and the possibility of MMP-9 being responsible for regulating CXCL12 protein levels, there was a decrease in MMP-9 mRNA expression in the peripheral blood on day 6 ($p > 0.05$ compared to normal control, Figure 4B).

Consistent with the observed increase in MMP-9 gene expression on day 6 in metaphyseal bone, MMP-9 protein expression in bone marrow supernatant specimens was found to be substantially increased on day 6 ($p < 0.001$ when compared to control) and then returned to basal levels on day

9, 10, 12 and 14 following MTX treatment (Figure 4C). On the other hand, there were no changes in MMP-9 inhibitor TIMP-1 protein expression in day 6 marrow samples when compared to control (Figure 4D).

Previously, *in vitro* studies have shown that MMP-9 present in mobilised bone marrow plasma completely abolished the presence of recombinant CXCL12 protein which can be blocked by an anti-MMP-9 monoclonal antibody (22). In the current study, in order to elucidate whether MMP-9 has a direct effect on CXCL12 protein expression, *in vitro* incubation of MMP-9 and CXCL12 followed by CXCL12 western blotting analysis revealed that the presence of recombinant MMP-9 protein reduced the amount of CXCL12 protein present following an 8 hour incubation period although this did not appear to abolish its immunoreactivity entirely when assessed by immunoblot. This suggests that increased MMP-9 expression observed following MTX treatment may be associated with the reduction in CXCL12 protein expression (Figure 4E).

CXCL12 does not appear to act to enhance cell differentiation. In view of the deregulated expression of the CXCL12/CXCR4 axis after MTX chemotherapy observed in our model, the potential role of CXCL12 in directly regulating haematopoietic or stromal precursor growth in *ex vivo* culture was assessed. Addition of recombinant CXCL12 to the growth medium of both MTX treated and untreated control BMMNCs did not obviously affect the formation of CFU-GM or CFU-F colonies when compared to BMMNCs grown in un-supplemented medium (Figure 5), suggesting that CXCL12 may not directly act as a promoting factor for the haematopoietic or stromal progenitor cell growth or differentiation.

Discussion

Cancer chemotherapy treatment has a myriad of detrimental side effects, including myelosuppression within the bone marrow (27-30), decreased bone formation and increased bone resorption, resulting in osteopenia or osteoporosis (41-42, 47). Intrinsic recovery of a damaged bone marrow environment is enabled by the maintenance of a quiescent stem cell pool residing at the endosteum, whereby these cells are induced into the cell cycle to allow re-establishment of a depleted marrow cavity. One important signalling pair that maintains quiescent stem cell populations and their regulated release into the cell cycle is the CXCL12/CXCR4 chemotactic axis. This study used a rat chemotherapy model with the commonly used antimetabolite methotrexate (MTX) to gain a better understanding of the actions of chemokine CXCL12 and receptor CXCR4 in the damage and ensuing recovery of the bone marrow microenvironment following chemotherapy treatment. Herein we illustrated that there appears to be deregulation of both CXCL12 and CXCR4 expression which is associated with a reduced bone marrow cellularity following MTX treatment. Furthermore, the reduced CXCL12 protein level following MTX damage occurs in parallel to MTX chemotherapy-induced MMP-9 expression. In addition, despite an overall reduction in total bone marrow cell viability on day 6 and day 9, there is an increase in haematopoietic differentiation potential.

MTX treatment was observed to cause an overall reduction in bone marrow cellularity, particularly on days 6 and 9, with a return to control density by day 14. During the damage and recovery time-course, there appears to be deregulation of the CXCL12/CXCR4 axis. Consistent with the reduced haematopoietic cellularity, haematopoietic cell CXCR4 mRNA expression was reduced in the BM non-adherent cell fraction on day 6 and day 9. This suggests that deregulation of CXCR4 is occurring in MTX chemotherapy-induced stress, potentially associated with the

reduced haematopoietic cell density observed in the BM cavity possibly as a result of the disruption of CXCL12/CXCR4 interaction (23-24).

Furthermore, associated with the reduced bone marrow cellularity, CXCL12 protein expression in both bone marrow and blood plasma were found to be altered over the time-course. While bone marrow CXCL12 protein concentration was reduced on day 9, despite its increased mRNA expression, blood plasma CXCL12 protein increased on day 6. These findings in the current MTX chemotherapy model suggest a potential role of the deregulated CXCL12/CXCR4 axis in mediating the reduced cellularity after MTX chemotherapy and confirm the previously demonstrated role of CXCL12 in G-CSF-induced mobilisation of haematopoietic progenitor cells out of the bone marrow microenvironment (22, 26).

Further investigations are required to elucidate the importance and underlying mechanism of deregulated CXCL12 in the present MTX chemotherapy model. However, the reduction in CXCL12 protein, yet increased mRNA expression observed in the bone marrow prompted investigations into the possibility of degradation or cleavage of CXCL12 protein in the bone marrow. Therefore we sought to investigate the potential involvement or role in our MTX chemotherapy model of matrix metalloprotease-9 (MMP-9) which has been illustrated to directly cleave CXCL12 at its N-terminus (22). In our MTX chemotherapy model, MMP-9 expression was found significantly increased on day 6 after MTX treatment both on the mRNA and protein levels in the bone marrow and yet expression of its natural inhibitor TIMP-1 was unaltered. In addition, our *in vitro* studies have demonstrated direct degrading potential of MMP-9 recombinant protein on CXCL12. These results suggest that the increase in MMP-9 protein expression in bone marrow specimens after MTX chemotherapy may be acting to degrade CXCL12 protein on day 9.

Further investigations into the association between MTX-induced bone marrow damage and MMP-9 up-regulation are required, particularly the relationship between increased osteoclast activity under stress-induced conditions potentially regulating MMP-9 protein expression (18-21). In addition, despite a clear reduction in bone marrow cellularity at the histological level, our *in vitro* haematopoietic progenitor cell differentiation assays showed a greater potential for CFU-GM differentiation on day 6. While this may be a recovery mechanism in place to re-establish the marrow environment following damage, further investigations into this increased differentiation capacity and the potential role of the CXCL12/CXCR4 axis in bone marrow progenitor cell recovery are essential.

In summary, our data illustrates that the gene and protein expression of the CXCL12/CXCR4 axis is altered during methotrexate chemotherapy and over the recovery period. Associated with the reduced bone marrow cellularity is decreased CXCR4 expression by haematopoietic cells and a reduction in CXCL12 protein in the bone marrow. Furthermore, consistent with previous studies showing MMP-9 can degrade CXCL12, increased expression of MMP-9 was observed in the bone and bone marrow after MTX chemotherapy. Our data suggests that altered CXCL12 and CXCR4 (due to the MMP-9 up-regulation followed by normalisation) during and following MTX chemotherapy treatment may play an important role in mediating the bone marrow damage and recovery. Further studies are required to elucidate any potential roles of MMP-9 deregulation in changes to the CXCL12/CXCR4 axis and in the damage and re-establishment of the bone marrow microenvironment associated with MTX chemotherapy. Defining the mechanisms governing regulation of the CXCL12/CXCR4 axis during chemotherapy-induced damage and recovery may reveal potential targets for preventing disruption to the axis and maintaining marrow microenvironment cell populations. Such knowledge may in turn improve the survival rate

and quality of life of cancer survivors in the long term, by reducing bone marrow toxicity and/or enhancing recovery and maintaining steady-state bone remodelling and turnover.

Figures
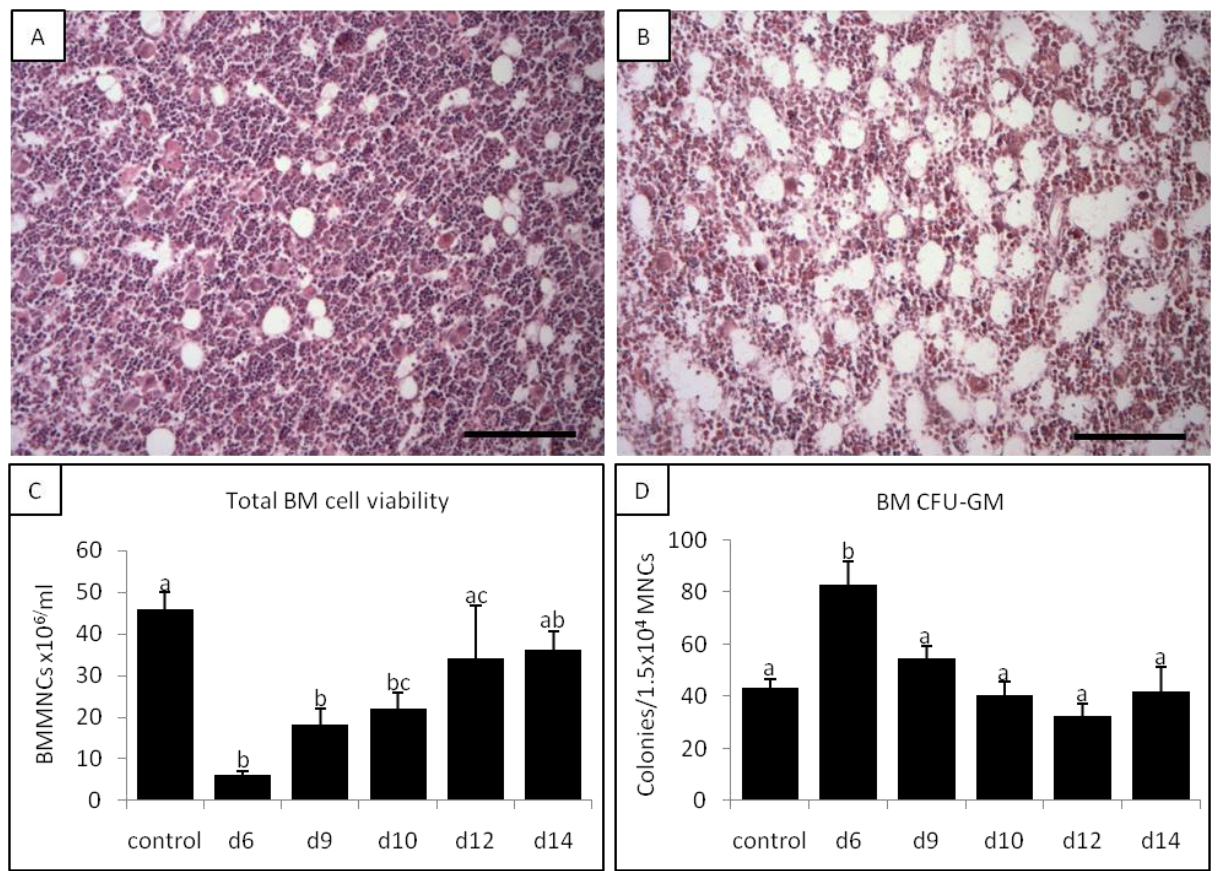
Figure 1: MTX-induced changes to bone marrow cellularity and recovery following short-term MTX treatment. A) H&E stained histology section of tibial diaphyseal bone marrow in a control rat. B) Histology of H&E stained section from a rat 6 days after initial dose of 0.75mg/kg MTX. Scale bar 100µM. C) Total bone marrow cellularity expressed as total mononuclear cells x10^6 cells/ml. D) *Ex vivo* granulocyte/macrophage-lineage colony formation (CFU-GM) with bone marrow cells isolated from rats over the MTX time-course. Different superscript letters denote means significantly different from each other p<0.05.
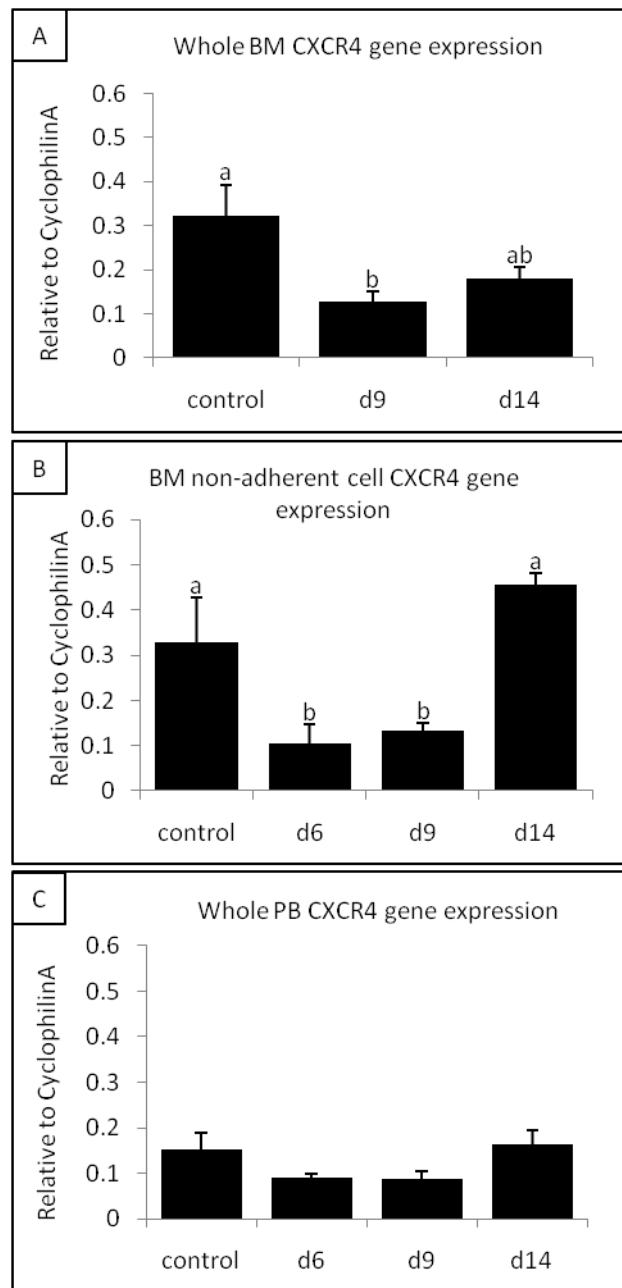
Figure 2: Changes in receptor CXCR4 mRNA expression over the MTX chemotherapy-induced damage/recovery time-course. Quantitative RT-PCR relative gene expression analysis relative to endogenous control Cyclophilin A with RNA isolated from whole bone marrow (A), bone marrow non-adherent cells (B), and whole peripheral blood specimens (C). Different superscript letters denote means significantly different from each other $p < 0.05$.
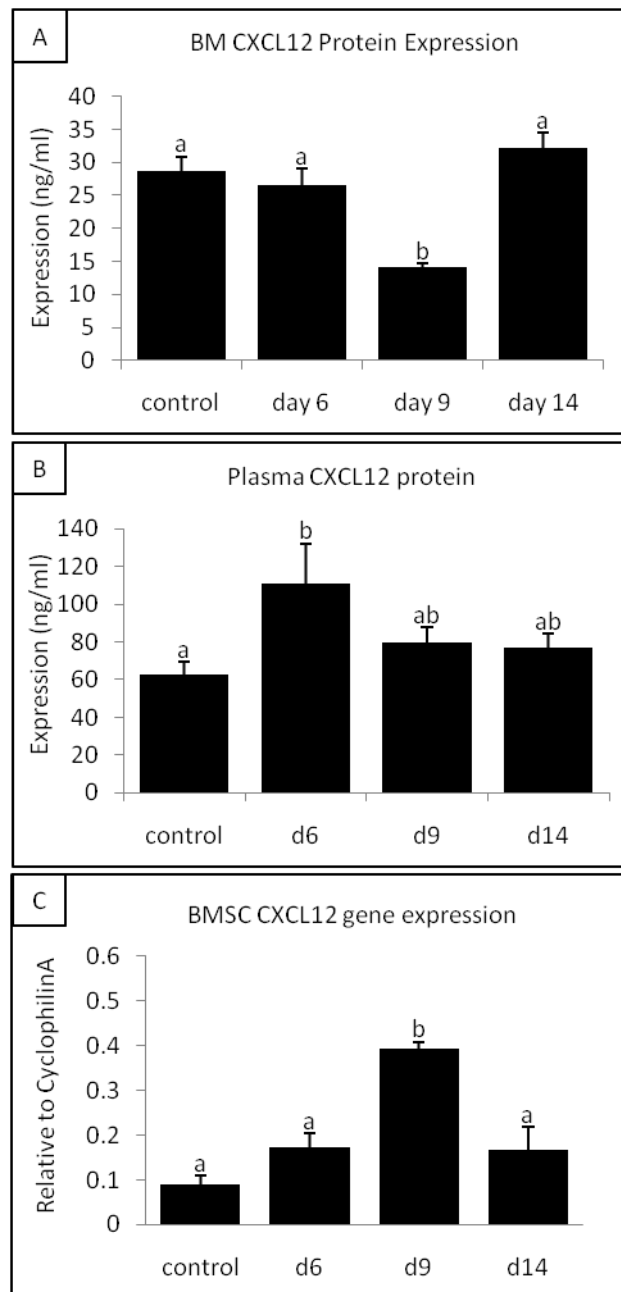
Figure 3: MTX chemotherapy-induced changes in chemokine CXCL12 protein and mRNA expression. CXCL12 protein levels (ng/ml) were determined by ELSIA in bone marrow supernatant (A) and in peripheral blood plasma (B) from MTX-treated and untreated control rats. C) Quantitative RT-PCR relative gene expression analysis of CXCL12 in RNA isolated from bone marrow stromal cells from normal and MTX-treated rats at different time points. Different superscript letters denote means significantly different from each other p<0.05.
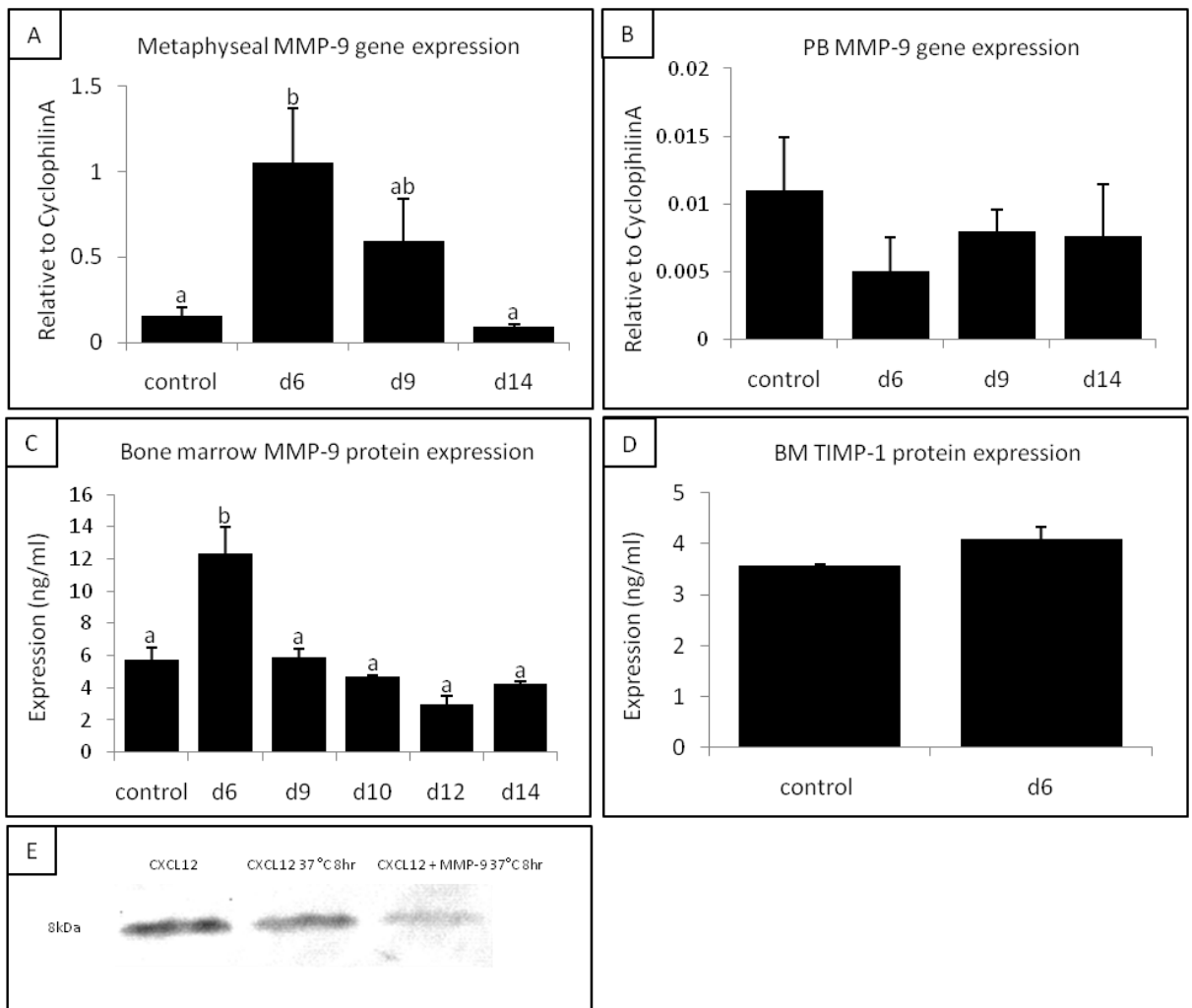
Figure 4: MTX chemotherapy-induced changes in MMP-9 and TIMP-1 expression and activity of MMP-9 in degrading recombinant CXCL12 *in vitro*. Quantitative RT-PCR relative gene expression analysis of MMP-9 in RNA isolated from whole metaphyseal bone (A) and from whole peripheral blood specimens (B). Protein levels (ng/ml) over the MTX time-course were determined by ELISA for MMP-9 (C) and for the naturally occurring MMP-9 inhibitor TIMP-1 (D) in bone marrow supernatant from control and day 6 after MTX treatment. E) Assessment by western blot, showing CXCL12 recombinant protein is partially degraded after *in vitro* incubation with MMP-9 recombinant protein for 8 hours. Different superscript letters denote means significantly different from each other p<0.05.

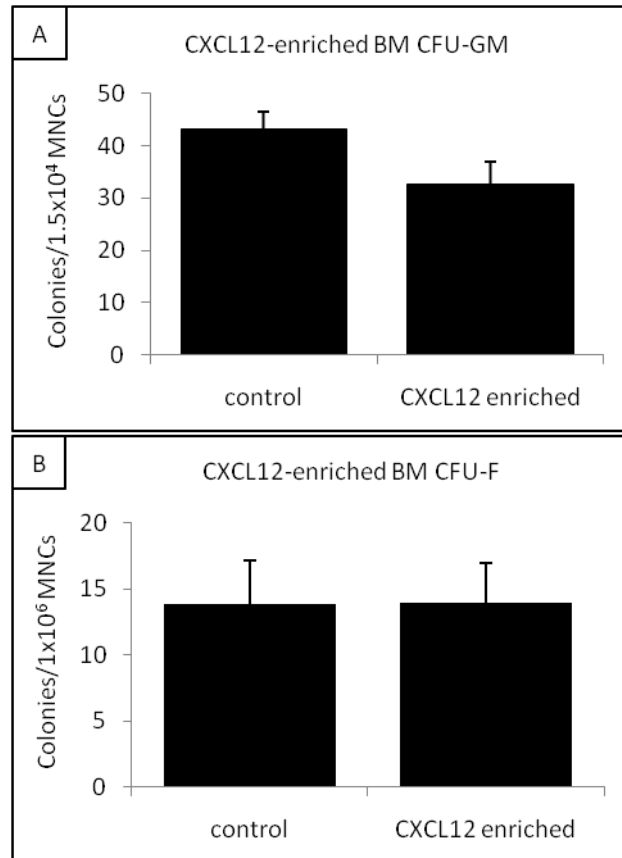**Figure 5:** Effects of exogenous CXCL12 protein on differentiation potential of bone marrow cells isolated from normal rats. A) *Ex vivo* granulocyte/macrophage-lineage colony formation assay; B) *Ex vivo* colony formation-fibroblast (CFU-F) assay.

Acknowledgments

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Chapter 5

General Discussion, Conclusions and Future Directions

The bone marrow microenvironment is home to mesenchymal and haematopoietic stem cells and their respective progeny, whereby interactions between cell types of each lineage maintains a steady-state functioning marrow. Regulation of maintenance of the quiescent storage niche residing at the bone marrow endosteum and appropriate lineage commitment and differentiation capacity are tightly regulated processes. Under conditions of stress or injury such as those induced by cancer chemotherapy, steady-state populations of cells and their interactions are disrupted and early precursor residents of the stem cell niche are induced to differentiate and migrate in order to repopulate the damaged microenvironment. This allows tissue-specific regeneration and subsequent bone marrow recovery, most notably evident following stem cell transplantation [24]. However, as cancer chemotherapy disrupts residents of the bone marrow [25-27], the initiated recovery mechanisms may potentially be dysfunctional or altered. Thus it is of great interest to elucidate the key cellular and molecular mechanisms involved in the regulation of steady-state and cancer chemotherapy-induced damage and subsequent recovery of bone marrow stem and progenitor cells and the associated bone and bone marrow defects including bone loss and marrow adiposity as well as marrow cell depletion. Therefore this PhD project aimed to illustrate the underlying mechanisms behind marrow microenvironmental damage and recovery, investigating the Wnt/β-catenin signalling pathway and the CXCL12/CXCR4 axis in an acute short-term rat model of methotrexate (MTX) chemotherapy treatment. Histological analyses were performed to demonstrate overall changes in cellular composition and trabecular bone and fat volumes within the marrow cavity and *ex vivo* and *in vitro* cell culture techniques were employed to investigate cellular commitment and differentiation capacity of marrow cells isolated from the treated rats over the time-course. In order to develop a comprehensive understanding of

the overall effects of MTX chemotherapy treatment on the bone marrow microenvironment and its function, protein and quantitative real time RT-PCR techniques were used to determine potential molecular and transcriptional expression changes occurring over the MTX-time-course. Furthermore, a synthetic inhibitor (BIO) for GSK-3β which is involved in regulating Wnt/β-catenin signalling was used to investigate the roles of this key pathway in the bone/fat switch observed after MTX chemotherapy. Lastly, the potential involvement of metalloproteinase MMP-9 was investigated associated with the reduced protein levels of CXCL12 in the bone marrow after MTX treatment.

Reduced bone mineral density is a common phenotype associated with cancer chemotherapy. For example, children suffering from acute lymphoblastic leukaemia (ALL) undergoing extensive chemotherapy treatment often present with disturbed bone metabolism following treatment regimens of multiple agents [28-30]. In an early clinical histological study, development of fat cells in the bone marrow of patients following intensive chemotherapy [31] was observed. In a recent study that examined DEXA scans and serum leptin levels in ALL patients, there was a predisposition towards osteopenia, excess adiposity and hyperleptinaemia in these patients [32]. MTX is an agent used commonly and typically in combination to treat patients with ALL. However, the association between this chemotherapy-induced reduction in BMD and an increased adipogenic marrow has not been investigated in a methotrexate model. Therefore in our current study, we have shown that MTX chemotherapy alone, in a short-term model of 0.75mg/kg MTX for 5-consecutive days over a time-course of 14 days, increases marrow adiposity, which is accompanied by a reduction in overall trabecular bone volume. This increase in marrow adiposity histologically is consistent with the significantly greater adipogenic potential of marrow stromal cells obtained from MTX-treated rats on days 6 and 9. Furthermore, gene

expression of key adipogenic players PPARγ and FABP4 from *ex vivo* cultured stromal cells increased on days 9 and 10, returning to normal by day 14.

As adipocytes and osteoblasts share the mesenchymal stem cell as a common precursor, osteogenic differentiation potential was also investigated, whereby differentiation capacity was reduced on day 9, followed by a return to control by day 14, suggesting that MTX treatment transiently alters the microenvironment to support fat formation at the expense of osteogenesis. This illustrates the potential existence of a reciprocal relationship between osteogenic and adipogenic differentiation of mesenchymal precursor cells in the MTX chemotherapy setting. This bone – fat switch has been reported earlier [33] and a switch in lineage commitment towards adipogenesis at the expense of osteogenesis under varying conditions [10-14]. Despite these reports, the mechanisms underlying our aforementioned finding on the switch in lineage commitment following MTX chemotherapy remain to be established.

As a means to identify key players in the MTX-induced damage to the bone marrow microenvironment and commitment to the adipogenic and osteogenic lineages, the Wnt signalling pathway was investigated. Wnt signalling is known to be essential for precursor cell differentiation down the osteoblastic lineage and in the regulation of the balance between adipogenesis and osteogenesis [34-36]. As the above changes in lineage commitment within the marrow microenvironment were persistently obvious over the course of MTX-induced damage, Wnt signalling genes were investigated in bone marrow stromal cells from MTX-treated rats over the time-course. Wnt-10b, a canonical Wnt ligand, has been defined as a stimulator of osteoblastogenesis and an inhibitor of adipogenesis [8-9, 34]. Wnt10b mRNA expression was found to be reduced during damage and returned to control by day 14. In this case, Wnt-10b may

be involved in re-establishing the normal balance of the bone microenvironment following MTX chemotherapy [37]. Furthermore, Wnt/β-catenin target gene survivin was reduced on days 6 and 9 in metaphyseal specimens from treated rats and similarly returned to control by day 14. This gene expression data suggests that MTX chemotherapy potentially reduced Wnt/β-catenin signalling in the bone marrow. Overall, the altered cell composition of the bone marrow cavity and changes in the Wnt/β-catenin signalling pathway over the MTX time-course suggests the possibility that altered Wnt signalling is involved in the enhanced adipogenic lineage commitment and decreased osteogenesis demonstrated following MTX chemotherapy. Thus, we hypothesised that a reduction in steady-state Wnt signalling following MTX treatment was a vital factor in increased adipogenic lineage commitment and the benefit of enhancing the signalling pathway would in turn enhance osteogenic potential and overall bone formation.

In order to target Wnt/β-catenin signalling and potentially up-regulate its function in the aforementioned MTX chemotherapy setting, the known agonist of Wnt/β-catenin signalling, the synthetic glycogen synthase kinase-3β (GSK-3β) inhibitor 6-bromoindirubin-3'-oxime (BIO) was administered to the rats treated with MTX. Previously, the use of BIO has shown promotion of mesenchymal differentiation potential towards the osteogenic but away from the adipogenic lineage [22-23]. Similarly, since glucocorticoids function to maintain GSK-3β levels and induce an up-regulation of the Wnt signalling inhibitor Dkk-1, in turn resulting in reduced bone formation [38], GSK-3β inhibition in an *in vivo* model has been demonstrated to limit glucocorticoid-induced bone loss in BIO-treated rats [23]. In the current study, BIO treatment alone did not alter stromal precursor cell lineage differentiation, however BIO concurrent treatment over the MTX time-course abrogated the MTX-induced reduction in bone volume, decrease in osteogenic differentiation capacity and down-regulated expression of osteogenic genes. BIO concurrent

treatment also prevented the previously identified MTX-induced increase in marrow adiposity, adipogenic differentiation potential of marrow stromal cells and expression of adipogenic genes. These findings suggest a potential role of GSK-3β inhibition in the control of microenvironmental cellular composition and can dampen the effects of MTX chemotherapy treatment on the osteogenesis to adipogenesis switch.

In addition, as MTX treatment in the present model altered myeloid composition of the marrow cavity and Wnt/β-catenin signalling plays a role in differentiation of the myeloid lineage, particularly granulocytes [39-40], differentiation capacity was assessed for the bone marrow haematopoietic cells from treated rats down the granulocyte-macrophage lineage (CFU-GM). While bone marrow MNC cellularity was reduced on days 6 and 11 after MTX chemotherapy, it was not changed by BIO treatment alone in the normal rats. However, CFU-GM differentiation potential was increased on day 6 following MTX and this increase was dampened by BIO supplementary treatment. Further investigations into the mechanism involved in such an altered differentiation potential following activation of Wnt signalling are required. However, it has been shown previously that constitutive activation of β-catenin in mouse models reduced differentiation of the myeloid lineage, yet compromised HSC maintenance by inducing cell cycling to erythroid progenitors, exhausting the HSC pool [40-41]. However due to differing models of β-catenin over-expression (β-catenin over-expression, LiCl addition, GSK-3β inhibition or addition of a Wnt ligand) and treatment schedule, haematopoietic responses are varied and contradictory, indicating that the influence of altered Wnt signalling on the haematopoietic cell population is not a simple matter. In addition, Wnt-10b has also been suggested to play an integral role in enhancing the growth of hematopoietic progenitors in the regenerative phase of bone marrow recovery [42]. Despite the varying haematopoietic responses elicited by altered Wnt signalling,

such changes clearly demonstrate that Wnt/β-catenin signalling is important in the maintenance of HSC proliferation and differentiation [42-44]. In our study it may be hypothesised that GSK-3β inhibition with BIO up-regulated Wnt signalling to cause a reduction in HSC differentiation potential after MTX chemotherapy, however the role of Wnt/β-catenin signalling in different cell populations within the bone marrow during recovery is varied. This also prompted interest in the possibility of alternative signalling mechanisms over the recovery phase of the bone marrow microenvironment following MTX-induced damage.

In order to address the potential involvement of an additional signalling mechanism in the recovery of the marrow cavity, the well documented chemotactic CXCL12/CXCR4 signalling axis was investigated. Intrinsic recovery of a damaged bone marrow environment is enabled by the maintenance of a quiescent stem cell pool residing at the endosteum, whereby these cells are induced into the cell cycle to allow re-establishment of a depleted marrow cavity [45-46]. One signalling pair involved in maintenance of the quiescent stem cell population and their regulated release into the cell cycle is the CXCL12/CXCR4 chemotactic axis [47-48]. CXCL12, expressed by stromal stem and progenitor cells, as well as vascular endothelial cells, has been illustrated to allow homing to the site of its up-regulation, enabling re-establishment of a damaged environment. It is also associated with maintenance of a quiescent stem cell population in endosteal regions within the bone marrow microenvironment [49]. Therefore, we hypothesised that deregulation of the CXCL12/CXCR4 interaction within the marrow microenvironment following damaging chemotherapy treatment may be associated with the damage and aid in the subsequent recovery observed in the present MTX model.

Consistent with the above short-term MTX-chemotherapy trials, there was an overall MTX-induced reduction in bone marrow cellularity, particularly on days 6 and 9, with a return to control density by day 14. As CXCR4 is largely present on cells of the haematopoietic lineage [50-51], in association with the reduced haematopoietic cellularity, haematopoietic cell CXCR4 mRNA expression was reduced in the BM non-adherent cell fraction on day 6 and day 9. This data indicates that deregulation of CXCR4 is apparent following MTX-induced stress, potentially associated with the reduced haematopoietic cell density observed in the BM cavity potentially as a result of the disruption of CXCL12/CXCR4 interaction [52-53].

As changes in the concentration of CXCL12 protein between the bone marrow and peripheral blood have been hypothesised to cause homing and migration of haematopoietic cells between sites [54], bone marrow and blood plasma CXCL12 protein concentrations were assessed in specimens from MTX-treated rats. In association with the reduction in bone marrow cellularity, bone marrow CXCL12 protein concentration was reduced on day 9, despite its increased mRNA expression and blood plasma CXCL12 protein was increased on day 6. Despite no clear relationship between expression changes in the two sites, these findings suggest deregulation of the CXCL12/CXCR4 axis. However, further investigations into the effects of MTX on potential bone marrow cellular mobilisation are required in order to determine how CXCL12 is involved in bone marrow damage and regeneration in our model. Previously, it has been demonstrated that CXCL12 played a role in G-CSF-induced mobilisation of haematopoietic progenitor cells out of the bone marrow microenvironment [54-55].

The reduction in CXCL12 protein, yet increased mRNA expression discussed above prompted investigations into the possibility of degradation or cleavage of CXCL12 protein in the bone

marrow. In order to investigate this, matrix metalloprotease-9 (MMP-9) was assessed in the present MTX model. MMP-9 is expressed largely by osteoclasts and has been previously identified to cleave CXCL12 at its N-terminus [55]. Following MTX treatment on day 6, MMP-9 gene and protein expression was found to be significantly increased in the bone marrow. While a significant change was observed on day 6, the natural MMP-9 inhibitor TIMP-1 protein expression in the bone marrow was also investigated and found to be unaltered, indicating that the increased MMP-9 protein level was not resultant of a reduction in its naturally occurring inhibitor. In addition, our *in vitro* studies indicate direct partial degrading potential of MMP-9 recombinant protein on CXCL12 protein. Increased MMP-9 protein within the bone marrow following MTX-treatment may potentially act to degrade CXCL12 protein, which was observed to decrease despite an increase in its mRNA expression as addressed above. However, MMP-9 activity must be addressed within the bone marrow following MTX treatment and its direct effect on CXCL12 must be demonstrated in the *in vivo* model. Furthermore, as MMP-9 is expressed by osteoclasts, further investigations into the relationship between MTX-induced MMP-9 up-regulation are required in association with a potential change in osteoclast activity following MTX treatment.

In summary, this PhD project employed a rat model of chemotherapy with the antimetabolite methotrexate (MTX) to investigate two key signalling mechanisms involved in the MTX-induced changes to bone/marrow fat volume and cellular composition of the bone marrow microenvironment over the damage and recovery time-course. Herein, MTX treatment was illustrated to cause an increase in adipogenic composition of the bone marrow cavity, in parallel to a reduction in trabecular bone volume. This is accompanied by an increased adipogenic, yet reduced osteogenic differentiation potential and related gene expression in bone marrow stromal

cells from treated rats. In addition, the adipogenesis to osteogenesis switch following MTX treatment is potentially associated with the observed changes in some Wnt signalling-associated genes, suggesting involvement of Wnt/β-catenin signalling in the above changes following MTX chemotherapy. Furthermore, the proposed switch in lineage commitment during the damage and recovery time-course following MTX was abrogated by concurrent treatment with the synthetic GSK-3β inhibitor 6-bromoindirubin-3'-oxime (BIO), an agonist of the Wnt/β-catenin signalling pathway. The damage and recovery potential of the bone marrow cell pool and re-establishment of the microenvironment were suggested to involve deregulation and regulation of the CXCL12/CXCR4 chemotactic axis. Steady-state functioning of this signalling pair is required for maintenance of a quiescence stem cell pool through interactions between the haematopoietic and stromal cell populations and a regulated release of its residents for appropriate repopulation of the bone marrow microenvironment. In the present model, MTX-induced changes to steady-state CXCL12 and CXCR4 expression were accompanied by an increase in CXCL12-degrading molecule MMP-9 and reduced CXCL12 protein concentration in the bone marrow. However, this was a transient event and re-establishment of marrow mononuclear cells and signalling components was apparent by day 14, further confirming the presence of a recovery phase following MTX treatment.

The data from this PhD project suggests that the Wnt signalling pathway is involved in the transient damage and recovery of the bone and bone marrow microenvironment, particularly associated with MSC lineage commitment, and that the CXCL12/CXCR4 signalling axis plays an important role in both the damage and re-establishment of the marrow mononuclear cell population.

Future Directions

This study illustrated a time-course of damage and recovery of the bone marrow microenvironment following acute short-term MTX chemotherapy and the involvement of the Wnt/β-catenin signalling pathway. This was further described using an agonist of the Wnt signalling pathway, BIO, to illustrate its potential role in the damage and recovery of cell populations within the bone marrow microenvironment. Of interest are further studies to investigate a chemotherapy regimen closely related to clinical combination therapy with multiple agents, in order to address the influence of Wnt signalling under more severe conditions. Furthermore a longer-term MTX dosing regimen would be important to address the long-term damage and altered recovery capacity that may be associated with the Wnt/β-catenin signalling pathway. As the Wnt signalling antagonist sFRP-1 was found to increase following short-term MTX treatment in a preliminary investigation, it would be of interest to further investigate its role in regulation of the Wnt/β-catenin signalling pathway in such a long-term *in vivo* study. In addition, the regulation of osteogenic/adipogenic balance over the MTX time-course with the use of BIO treatment following a different treatment schedule or dosing regimen may more effectively target Wnt/β-catenin signalling as a potential therapeutic target to prevent bone loss and marrow adiposity in the present and in a longer-term chemotherapy model.

Deregulation of the CXCL12/CXCR4 signalling axis following MTX chemotherapy was illustrated over the time-course and its potential involvement in re-establishment of the bone marrow microenvironment was hypothesised. It is important to next clarify the direct role of CXCL12/CXCR4 signalling in the mobilisation of haematopoietic cells out of the marrow following MTX treatment, disruption of the endosteal niche by movement of stem cells into the cell cycle and furthermore, the homing of CXCR4-expressing haematopoietic cells back to the marrow

environment in order to repopulate the depleted marrow cavity. Firstly, the migration capacity of CXCR4 expressing cells isolated from MTX treated and untreated rats to recombinant CXCL12 using a transwell assay would be an ideal preliminary investigation of whether treated cells have a disrupted migratory capacity. In addition, the degrading potential of MMP-9 causing disruption to such chemotactic potential is an issue that needs to be addressed and examined to see whether this may also be a potential target for future therapies. Similarly using a transwell set-up, migration of isolated cells to CXCL12 alone or CXCL12 incubated with MMP-9 to assess the extent of degradation would be an important preliminary investigation. Furthermore, isolated control cell migration to *ex vivo* isolated bone marrow of treated and untreated rats assessed against control marrow incubated with or without MMP-9 recombinant protein may clarify the role MMP-9 plays when mimicking a simple *in vivo* setting *ex vivo*. These preliminary assays would lead on to investigations of more complex *in vivo* systems; viewing green-fluorescently labelled CXCR4-expressing cell movement into or out of the bone marrow microenvironment following MTX chemotherapy treatment and fluorescent visualisation of the interaction of CXCL12-expressing and CXCR4-expressing cells at the endosteum before and after MTX treatment to illustrate the importance of and disruption to the axis in our model.

Gaining a broader understanding of the mechanisms involved in both the damage and recovery of bone marrow cell populations following MTX and alternative treatments that induce a similar phenotype will enable development of targeted therapies. Combination treatments targeting both the damage and recovery phase of the bone marrow microenvironment following chemotherapy may improve treatment strategies for chemotherapy-induced osteoporosis, marrow adiposity and myeloid depletion apparent in the bone marrow cavity.

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