Roles of chemokine CXCL12 in bone growth defects induced by methotrexate and dexamethasone chemotherapeutic treatment

A THESIS SUBMITTED IN TOTAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTOR OF PHILOSOPHY

by

QIAN TANG

School of Pharmacy and Medical Sciences,

Division of Health Sciences,

University of South Australia, Adelaide, SA, Australia.



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TABLE OF ABBREVIATIONS

ALL	Acute lymphoblastic leukemia
ALP	Alkaline phosphatase
APC	Adenomatous polypsis coli
BMD	Bone mineral density
BMP	Bone morphogenetic protein
C/EBPα	CCAAT/enhancer binding protein alpha
CK1	Casein kinase 1
CT	Calcitonin
Dex	Dexamethasone
Dkk-1	Dickkopf-1
DMP-1	Dentin matrix acidic phosphoprotein-1
ECM	Extracellular matrix
FABP4	Fatty acid binding protein 4
FGF-23	Fibroblast growth factor-23
GBP	Glycogen synthase kinase-3β binding protein
GC	Glucocorticoid
GH	Growth hormone
GHBP	Growth hormone binding protein
GHR	Growth hormone receptor
GSK-3β	Glycogen synthase kinase-3β
HSC	Hematopoietic stem cell
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
Ihh	Indian hedgehog
IL	Interleukin
LRP5/6	Lipoprotein receptor-related protein 5/6

M-CSF	Macrophage colony-stimulating factor
MEPE	Matrix extracellular phosphoglycoprotein
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
MTX	Methotrexate
NCP	Non-collagenous protein
OCN	Osteocalcin
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
OSCAR	Osteoclast-associated receptor
Osx	Osterix
PG	Proteoglycan
PHEX	Phosphate-regulating neutral endopeptidase
PPARγ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
RANKL	Receptor activator of nuclear factor-κB ligand
Runx2	Runt-related transcription factor 2
SFRP1	Secreted frizzled-related protein-1
T3	Triiodothyronine
T4	Thyroxine
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TR	Thyroid hormone receptor
TRAP	Tartrate-resistant acid phosphatase

THESIS SUMMARY

Methotrexate (MTX) and/or dexamethasone (Dex) are the two most commonly and intensively used agents in childhood oncology, particularly in acute lymphoblastic leukemia (ALL). However, intensive use of these chemotherapeutic agents is known to cause bone growth defects such as growth arrest, bone loss and fracture. Although the mechanisms for chemotherapy-induced bone growth defects still remain unclear, significant disruptions to cell population and interaction such as induced chondrocyte/osteocyte apoptosis accompanied by enhanced chondroclast/osteoclast recruitment and activity have been observed within bone and marrow microenvironment. However, potential molecular signals for the link between skeletal apoptosis and chondroclast/osteoclast recruitment are unclear. The current study addressed the potential roles of specific cytokine(s) and/or chemokine(s) induced by apoptotic chondrocytes/osteocytes in the chondroclast/osteoclast recruitment and bone defects following MTX and/or Dex treatment *in vitro* and *in vivo*.

As the first step, the current study utilized *in vitro* MTX and/or Dex treatment models to determine whether treatment-induced apoptotic chondrocytes/osteocytes secrete specific cytokine(s)/chemokine(s) with the capacity to stimulate osteoclast recruitment and differentiation. After 48 h 10^{-5}M MTX and/or 10^{-6}M Dex treatment in cultured mature ATDC5 chondrocytes/ mature IDG-SW3 mature osteocytes, significantly induced cell apoptosis was observed. PCR array analyses of MTX $\pm$ Dex-treated chondrocytes/osteocytes identified that chemokine CXCL12 exhibited the most prominent induction in each treatment group, which was confirmed by quantitative RT-PCR analysis in treated cells and ELISA for the conditioned medium from treated cells. In addition, the conditioned medium from the

treated chondrocytes/osteocytes was found to enhance migration of RAW264.7 osteoclast precursor cells and their osteoclast formation. In particular, the promoting effect of the conditioned medium was significantly inhibited by the presence of the neutralizing antibody for CXCL12. These findings suggested that CXCL12 derived from apoptotic chondrocytes/osteocytes following MTX and/or Dex treatment could be an important osteoclastogenesis stimulating factor, which is associated with aggravated cartilage/bone loss.

The current study subsequently investigated the potential effects of chemokine CXCL12 blockade in preventing bone defects in rats caused by Dex treatment. Male young rats were orally gavaged daily with Dex at 1 mg/kg/day for consecutive 14 days to mimic the clinical regimen for acute ALL maintenance treatment; while half of the rats received supplementary intraperitoneal injection of anti-CXCL12 neutralizing antibody (1 mg/kg) three times weekly for CXCL12 loss-of-function studies. Dex treatment was shown to cause an obvious reduction in growth plate thickness, primary spongiosa height, trabecular bone volume, which were associated with induced chondrocyte/osteocyte apoptosis, enhanced chondroclast/osteoclast recruitment, stimulated osteoclastogenic differentiation potential, marginally inhibited osteoblastogenic differentiation potential and promoted bone marrow adipogenic differentiation potential. CXCL12 was found to be obviously induced in apoptotic growth plate chondrocytes and metaphyseal bone osteocytes. Blocking CXCL12 function by the supplementation of the anti-CXCL12 neutralizing antibody was observed to significantly prevent Dex-induced growth plate cartilage and bone loss. Thus, these findings confirmed *in vivo* the role of CXCL12 as a molecular link between Dex treatment-induced skeletal cell apoptosis and chondroclastic/osteoclastic recruitment and growth plate cartilage/bone loss,

and has revealed a therapeutic potential of blocking CXCL12 function in preventing bone growth defects during/after Dex treatment.

DECLARATION

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; to the best of my knowledge it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications, are clearly acknowledged.

Signed _____ [redacted] _____ Date 07/09/17

Qian Tang

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CHAPTER ONE

Literature review & Project aims

1.1 Introduction to Literature Review

Although chemotherapy has achieved great success in treating paediatric malignancies, the intensifying use of chemotherapeutic agents has severe adverse effects on bone growth in childhood cancer patients (Xian et al., 2007). These defects have been associated with altered regulation of the cells residing in the bone marrow microenvironment and the bone itself , for which the mechanisms remain unclear (Fan et al., 2012a). This PhD project will address the potential functions and underlying mechanisms of skeletal cell death in childhood cancer chemotherapy-induced bone growth defects.

1.2 Skeletal Physiology

1.2.1 Function and Classification of Bones

The skeletal system composed of bones, cartilages, joints, and ligaments, accounting for about 20% of the body mass, within which bones make up most of the skeleton (Marieb and Hoehn, 2007). Bone is termed as a highly specialized framework of the body, distinguished by its rigidity, hardness, and potential for regeneration and self-repair (Kini and Nandeesh, 2012). Normal adult human skeleton consists of 206 bones (Gray and Lewis, 2000). According to their shape, these bones are generally divided into four types, namely long, short, flat and irregular bones (Fig.1-1) (Clarke, 2008). A long bone is defined as longer than it is wide (long cylinder-like shape); a short bone appears approximately equal in length, width and thickness (cube-like shape); a flat bone is generally thin and curved (plate-like shape); and irregular bones have a complicated, peculiar shape and cannot be grouped into the categories mentioned above.

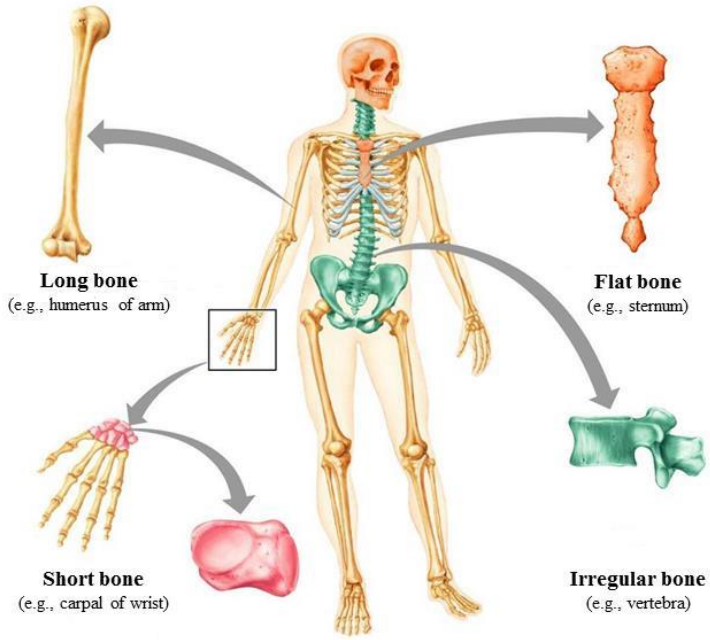


Figure 1-1. Classification of bones. Four different bone categories are classified on the basis of their shapes: long, short, flat and irregular bones. An example of each category is illustrated. Figure was adapted from (Marieb and Hoehn, 2007).

The bones of the skeleton serve a variety of functions: they protect vital internal organs, provide levers for the muscles to permit movement and locomotion, endow the environment for hematopoiesis and adipogenesis within bone marrow, take part in mineral homeostasis and acid-base equilibrium, and also act as the reservoir for stem cells, cytokines, chemokines and growth factors (Taichman, 2005). Recent investigations have also considered bone as an endocrine organ that modulates glucose metabolism and energy homeostasis (Oldknow et al., 2015).

1.2.2 Gross Anatomy of Bones

The human skeleton has a complex macro and micro structure. Each long bone is composed of a diaphysis, two epiphyses and two metaphyses (Fig.1-2) (Currey, 2002). Diaphysis is the hollow shaft of a long bone, whilst the epiphyses are the ends of the bone. The region

between the epiphysis and diaphysis is defined as the metaphysis. The growth plate is a hyaline cartilage plate within the metaphyseal region at both ends of the long bone in children and adolescents. The growth plate eventually fuses and is replaced by an epiphyseal line during late stage of adolescence (Teti, 2011). The medullary cavity is the central cavity of a long bone to store bone marrow and is lined with a thin membrane called endosteum. Periosteum is a double-layered membrane which is on the exterior surface of the bone except the joint surfaces (Fawcett, 1994). The joint surface of epiphyses is covered by articular cartilage which is a thin layer of hyaline cartilage (Kini and Nandeesh, 2012).

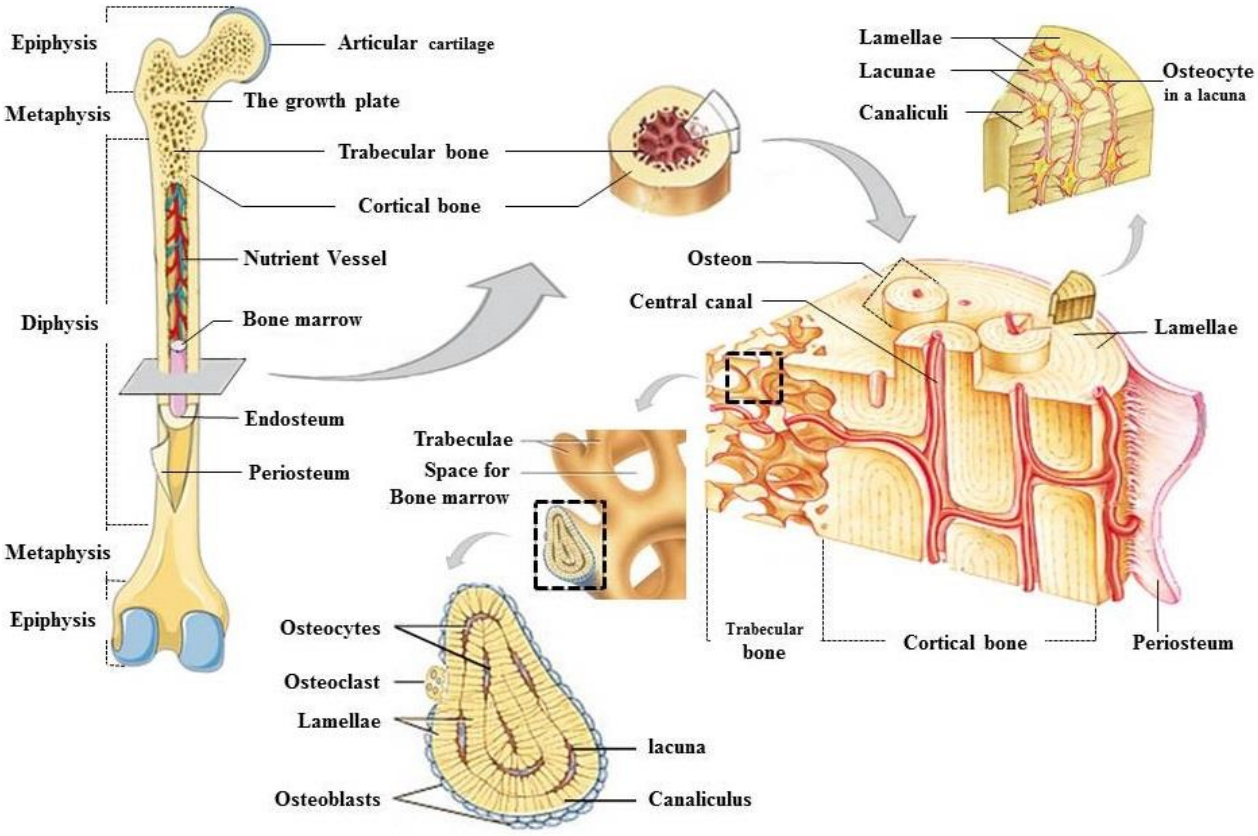


Figure 1-2. Structural organization of a long bone. The long bone allows for the best visualization of all of the parts of the bone. The long bone consists of a diaphysis, two epiphyses and two metaphyses. Cortical bone is composed of dense and parallel arranged osteons providing the hard exterior of the bone. Trabecular bone consists of interconnecting meshwork of bony trabeculae with low density and strength but high surface area, harboring the medullary cavity inside the bone. Osteocytes reside in lacunae within bone matrix and interconnect via canaliculi. In addition, bone is pervaded with blood vessels and nerves. Figure was adapted from (Marieb and Hoehn, 2007).

In addition, depending on the texture and overall structure, total bone mass of an adult skeleton consists of two different components: cortical bone (the outer part) makes up 80% of bone mass and the remaining 20% is composed of trabecular bone (the inner part) (Clarke, 2008). The ratios of cortical to trabecular bone are altered in different categories of bone and skeletal sites (Ralston, 2009). Cortical bone is dense, compact, and homogeneous, which constitutes the outer part of the skeletal structures and forms an envelope around the medullary cavity with an external periosteal surface and internal endosteal surface (Fig.1-2) (Ralston, 2009). Its major function is to provide mechanical strength and protection and it is mainly observed in the shaft of long bones (Ruimerman, 2005). Cortical bone is structurally arranged in overlapping parallel and small functional units termed osteons (Clarke, 2008). Osteons, also known as the Haversian system, refers to a central canal containing longitudinally oriented vessels and associated osteocytes (the major type of bone cells) surrounded by concentric lamellae of bone matrix (Shapiro, 2008). Compared to cortical bone, trabecular bone has a lower density and consists of an interconnecting honeycomb-like meshwork of thin wires called trabeculae, interspersed in the bone marrow compartment (Fig.1-2) (Clarke, 2008). It is principally observed at the end of long bones, near joint surfaces, within vertebrae, and in the inner portions of the pelvis and other large flat bones (Ruimerman, 2005). Trabecular bone also has much higher surface area than cortical bone so that it is typically more metabolically active and has the potential to provide the initial supplies of mineral in acute deficiency states (Ralston, 2009). Thus, bone is remodeled (turnovered) more rapidly from sites rich in trabecular bone under conditions of increased bone turnover.

In comparison with long bones, short, flat and irregular bones have no diaphysis or epiphyses. They consist of periosteum-covered cortical bone and endosteum-covered trabecular bone. There is also no medullary cavity found in short, flat and irregular bones, although bone marrow is still contained between the trabeculae (MariBb, 2006).

1.2.3 Bone Composition

Bone is a mineralized connective tissue and composed of specialized bone cells as well as mineralized and unmineralized extracellular matrix (ECM) (Tab.1-1) (Currey, 2002).

Table 1-1. The components of bones

Bone cells	• Osteoblasts • Osteocytes • Osteoclasts
Extracellular matrix	• Organic portion Collagen, Proteoglycans, Non-collagenous proteins • Inorganic portion Calcium and phosphorus

Bone cells are described as a highly complex organic structure, whereas ECM is the intercellular substance made up of organic and inorganic components (Currey, 2002). Three brief types of cells are observed within bone tissue including bone-forming osteoblasts, bone-resorbing osteoclasts and mechanosensory osteocytes (Fig.1-2) (Marieb and Hoehn, 2007). Osteoid, the unmineralized and organic portion of bone matrix, is formed prior to the maturation of bone tissue (Kini and Nandeesh, 2012). It consists of collagenous fibers (primarily type I collagen), proteoglycans (PGs), and non-collagenous proteins (NCPs) such as osteocalcin (OCN) and osteonectin (ON) (Sims and Johnson, 2012). On other hand, the inorganic portion of ECM is principally comprised of calcium and phosphorus in the pattern

of tiny and compactly packed crystals in and around the collagen fibers within ECM, termed as hydroxyapatite crystals (Currey, 2002). Intracellular communications and cell-matrix interactions are confirmed to play vital roles in the stimulation, maturation and activation of the bone cells.

1.2.4 Bone Cells

Although bone cells only comprise 2% of the bone volume, they are critical to bone development and function (Currey, 2002). It is well established that there are two stem cell types and their progenies residing in the bone marrow, which are mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). Both of them are maintained in self-renewing compartments and differentiate into multiple bone cell lineages such as osteoblasts and osteoclasts, respectively (Fig.1-3) (Bonfield and Caplan, 2010).

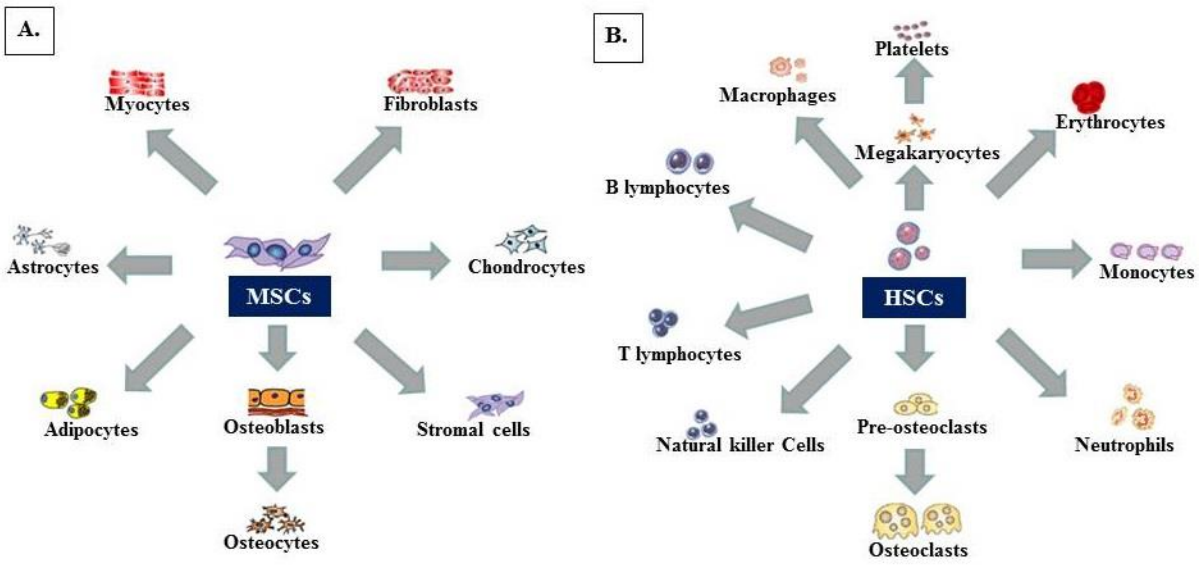


Figure 1-3. Differentiation of mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs). A. MSCs are capable of differentiating into a variety of phenotypes such as chondrocytes, osteoblasts and adipocytes. **B.** HSCs can differentiate into osteoclasts and other blood cell types. Figure was adapted from (Bonfield and Caplan, 2010).

1.2.4.1 Osteoblast function and differentiation

Osteoblasts are fully differentiated cells derived from MSCs and locate on the surface of the osteoid. They function principally secreting collagenous bone matrix as well as managing its subsequent mineralization, resulting in establishment of new layer of bone (Marieb and Hoehn, 2007). Osteoblast differentiation is a tightly regulated process beginning with the commitment of MSCs towards osteoprogenitor cells, after which further develop to pre-osteoblasts and to mature functional osteoblasts (Fig.1-4) (Marie, 2008). Osteoblasts are shown to selectively secrete different types of collagens and NCPs during the differentiation process. In the early stage, committed pre-osteoblasts acquire the ability to express alkaline phosphatase (ALP) which is an enzyme vital to initiation and regulation of mineralization, and type I collagen which is the major organic component of the ECM (Fakhry et al., 2013). Mature osteoblasts are identified to be highly enriched in ALP and also secrete specific NCPs such as OCN (Blum et al., 2001). The expression levels of ALP are upregulated based on matrix deposition and osteoblast maturation, but decline at terminal differentiation of osteoblasts to osteocytes (Rucci, 2008). Thus ALP could be considered a molecular marker for the early stage of osteoblastogenesis (Fig.1-4). On the other hand, OCN is most abundant NCP of bone matrix and exert the promoting effects on bone formation and mineralization (Zoch et al., 2016). The upregulation of OCN expression levels is observed particularly in post-proliferative mature osteoblasts following osteoid mineralization (Nakashima and de Crombrugghe, 2003). Thus OCN could serve as a marker for the late stage of osteoblast differentiation (Fig.1-4). The average lifespan of human osteoblasts is approximately 3 months (Manolagas, 2000). At the end phase, around 60 to 80% osteoblasts underdo apoptosis and part of the remaining osteoblasts are embedded in lacunae of the mineralized matrix individually and further

differentiate into osteocytes, while the rest mature osteoblasts become flat-shaped bone-lining cells extended over quiescent (non-remodeling) bone surfaces (Fig.1-4) (Long, 2012). The connection between the bone-lining cells and the osteocytes is vital for the activation signal transmission during bone remodeling (Arboleya and Castaneda, 2013).

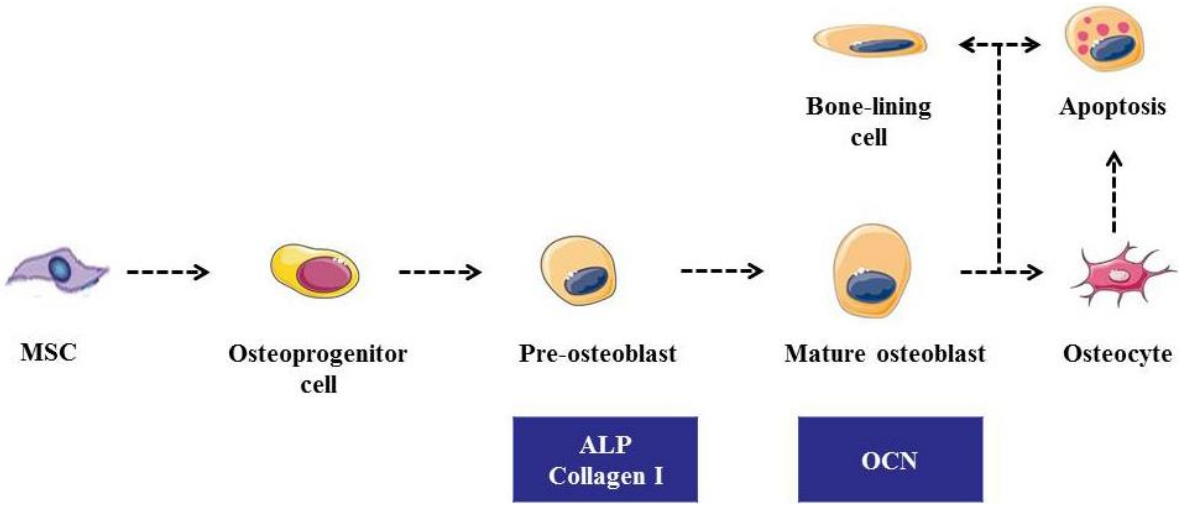


Figure 1-4. Schematic illustration of osteoblast differentiation and selective molecular markers. The different stages of osteogenesis are related to some unique and altered expressions of specific molecules which are shown in blue textboxes. Figure was adapted from (Arboleya and Castaneda, 2013).

1.2.4.2 Osteocyte function and differentiation

As stated before, following mineralization of the surrounding matrix, a subset of osteoblasts are embedded within the bone matrix, undergo changes in structure and differentiate into osteocytes (Schaffler and Kennedy, 2012). Osteocytes are the most abundant cellular component of bones which comprises 90 % to 95 % of total bone cells (Franz‐Odendaal et al., 2006). As the terminally differentiated cells, osteocytes are non-proliferative and have totally altered structure and function (Schaffler and Kennedy, 2012). Each osteocyte is located in an ellipsoidal space within the calcified matrix, known as a lacuna. They also have a meshwork of filipodial processes that extend through canaliculi, the tiny cylindrical channels

within the bone matrix, enabling them to receive nutrients and communicate with each other and other cell types (Fig.1-5) (Franz‐Odendaal et al., 2006).

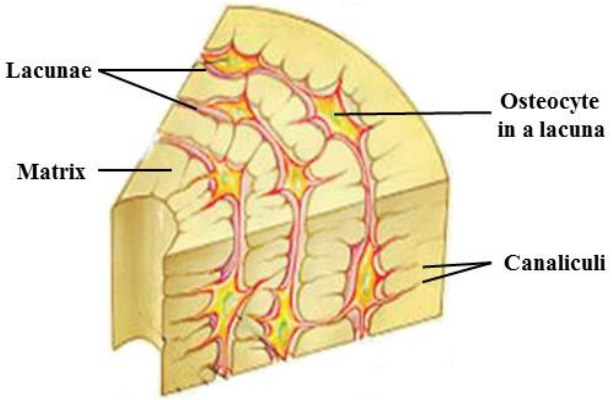


Figure 1-5. Osteocytes in lacunae. Osteocytes reside in lacunae within calcified bone matrix and interconnect via canaliculi. Figure was adapted from (Marieb and Hoehn, 2007).

Although the overall functions of osteocyte are still not fully understood, osteocytes have been demonstrated to function within a mechanosensory network to support bone structure and metabolism (Clarke, 2008). It has been revealed that osteocytes have the capacity to sense mechanical strain and then translate the forces into biochemical signals, resulting in the structurally functional adaptation of bones (Lanyon, 1993). In particular, osteocytes are known to play key roles in coordinating bone remodeling through expression and secretion of molecules including sclerostin and receptor activator of nuclear factor-κB ligand (RANKL) (see below) that regulate formation and activities of osteoblasts and osteoclasts (Nakashima et al., 2011; O'Brien et al., 2013; Xiong et al., 2011). Sclerostin, a secreted glycoprotein encoded by the SOST gene, is the negative regulator of osteoblast differentiation and bone formation due to its antagonizing effects on receptor LRP5/6 in canonical Wnt/β-catenin signaling (Canalis et al., 2007). Sclerostin is observed to be highly expressed by terminally differentiated cells deeply embedded in mineralized matrix and thereby is considered as a mature osteocyte phenotype marker (Fig.1-6) (Poole et al., 2005). It is also evident that

osteocytes could regulate local and systemic mineralization. Three types of proteins including phosphate-regulating neutral endopeptidase (PHEX), matrix extracellular phosphoglycoprotein (MEPE) and dentin matrix acidic phosphoprotein-1(DMP-1) are identified to be expressed by osteocytes to develop and maintain bone matrix during the mineralization process (Jacobs et al., 2010; Kramer et al., 2010). Meanwhile, the transportation of inorganic minerals within the skeleton essentially requires interface with other organs, particularly the kidneys. Fibroblast growth factor-23 (FGF-23), the growth factor vital to the coordination of the bone-kidney axis, is confirmed to be predominantly produced by osteocytes and regulated by PHEX, MEPE, and DMP-1 (Crofton et al., 2000). Therefore, osteocytes could coordinate systemic mineralization via renal regulation of systemic phosphate homeostasis, possibly by FGF-23 (MariBb, 2006). The upregulations of PHEX, MEPE, DMP-1 and FGF-23 are consequently demonstrated to represent a signature for transition from osteoblasts to osteocytes. Aside from above factors, more osteogenesis-selective biomarkers which play unique regulatory roles in altering osteocyte morphology and function during osteocyte differentiation have been identified. E11, also known as GP38, a membrane-bound protein prominently expressed by the early embedding osteoid osteocytes, plays an essential role in the formation of dendrites in osteocytes and is considered as the earliest osteocyte marker (Fig.1-6) (Dallas et al., 2013).

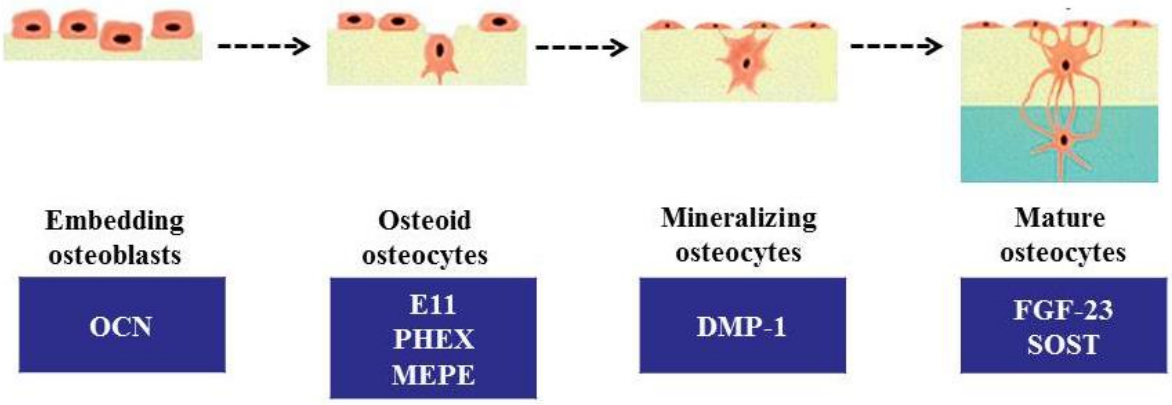


Figure 1-6. Schematic illustration of osteocyte differentiation and selective molecular markers. The different stages of osteogenesis are related to some unique and altered expressions of specific molecules which are shown in blue textboxes. Figure was adapted from (Bonewald, 2011).

Although it is well accepted that the mature osteocytes represent the terminally differentiated osteoblast lineage cells, osteocytes have been shown to be with a long lifespan and only about 2.5% osteocytes undergo apoptosis each year under physiologic conditions (Manolagas and Parfitt, 2010). The normal rate of osteocyte apoptosis is confirmed to be essential for skeletal health (Jilka et al., 2014). However, emerging evidence has revealed that external influences such as aging, pausimenia, under unloading, and microdamage are responsible for the induction of osteocyte apoptosis, leading to the increased skeletal fragility (Jilka et al., 2007; Su et al., 2017b; Weinstein and Manolagas, 2000). In particular, previous *in vivo* studies have shown that intensive use of some chemotherapeutic agents could considerably promote osteocyte apoptosis, which is associated with the bone growth defects (Xian et al., 2008; Xian et al., 2007). Most importantly, a tight temporal-spatial coupling between the accumulation of apoptotic osteocytes and the increase in recruitment of osteoclasts has also been illustrated (Bellido, 2014; Kennedy et al., 2012; Plotkin, 2014; Shandala et al., 2012). Although the precise mechanisms still remain largely unknown, it is indicated that apoptotic osteocytes have the potential in expressing specific regulatory factors to regulate osteoclast precursor cell

recruitment and osteoclast formation and activity. Thus, osteocytes are vital in keeping the balance between bone formation and resorption, as well as maintaining phosphate homeostasis and mineral metabolism. Especially, apoptotic osteocytes may be a potent osteoclastogenesis inducer.

1.2.4.3 Osteoclast differentiation and function

Different to osteoblasts and osteocytes, osteoclasts are differentiated from bone marrow monocyte/macrophage lineage cells derived from HSCs (Fig.1-3) (Bonfield and Caplan, 2010). They are classified as exclusive bone-resorbing cells which can create an acidic environment and produce collagen-degrading enzymes to remove the mineralized matrix and disintegrate the organic bone (Weinstein, 2012). The process of osteoclast differentiation is characterized by a sequence of events including stem cell division, differentiation into mononuclear precursors, migration to the sites of bone resorption, fusion to multinucleated osteoclasts, and activation to bone resorbing osteoclasts (Fig.1-7) (Teitelbaum, 2000). It has been well-established that intercellular communication plays a crucial role in osteoclastogenesis. In particular, RANKL pathway is shown to be mandatory for the differentiation and activation of osteoclasts (Weinstein, 2012). RANKL, which is a type II membrane protein belonged the tumour necrosis factor (TNF) cytokine family and expressed on the surface of osteoblasts, is capable of binding to its cell-surface receptor RANK, which is a type I membrane protein expressed on nearby osteoclast precursors as well as osteoclast themselves (Quinn et al., 2002). The RANKL-RANK interaction and downstream signaling cascades (e.g. NF-κB and NFATc1) could in turn activate the gene expression of osteoclastogenic regulators such as osteoclast-associated receptor (OSCAR), tartrate resistant

acid phosphatase (TRAP) and cathepsin K to stimulate the differentiation and activity of osteoclasts, triggering in the subsequent bone resorption (Boyle et al., 2003). On the other hand, osteoprotegerin (OPG), a soluble cytokine receptor belonging to TNF receptor superfamily and mainly produced by osteoblast lineage cells, is found to be a potent inhibitor for osteoclast formation (Weinstein, 2012). As a soluble decoy receptor for RANKL, OPG competitively binds to RANKL so that the interaction between RANKL and RANK could be significantly disrupted (Rauner et al., 2007). Thus, the RANKL/OPG ratio is the determinant for osteoclastogenesis.

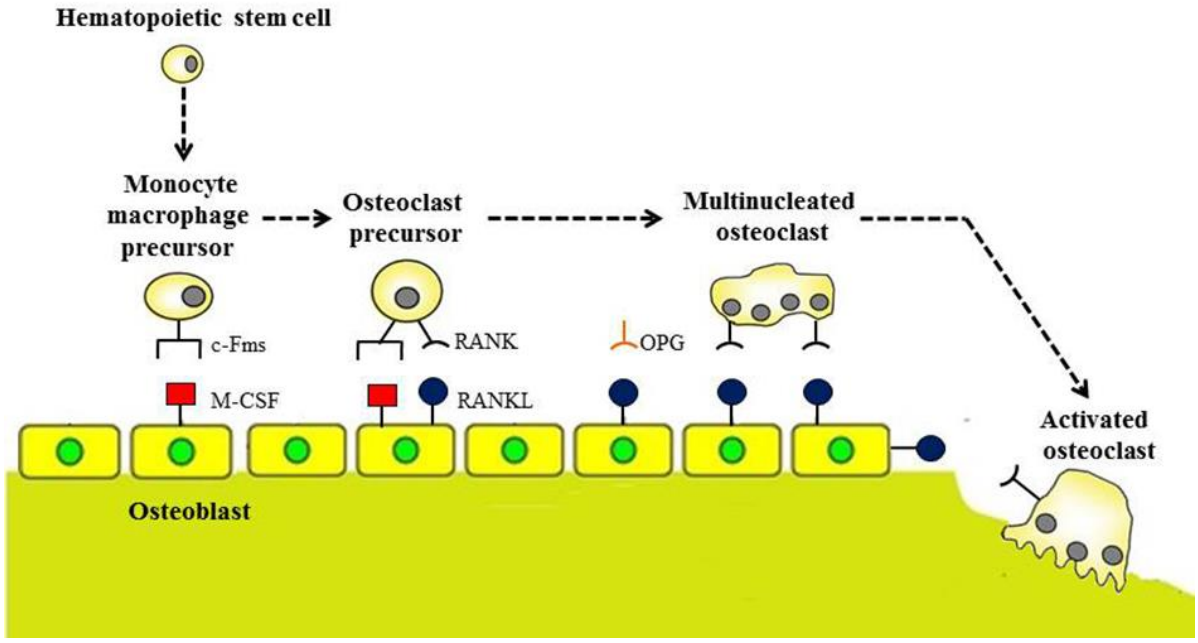


Figure 1-7. Schematic illustration of osteoclast differentiation and major regulators. Cross-cell talk between osteoclast precursor and osteoblast or its stromal cell precursor is a requisite for osteoclast differentiation. Osteoblast or stromal cell precursor express two key osteoclastogenic cytokines: RANKL and M-CSF. Meanwhile their receptors are expressed by osteoclast precursor. OPG is a soluble decoy receptor for RANKL. Figure was adopted from (Senba et al., 2012).

In addition, macrophage colony-stimulating factor (M-CSF) is another secreted cytokine released by osteoblasts and has been illustrated to be key regulator for osteoclast

differentiation (Fixe and Praloran, 1998). By interacting with its membrane receptor (c-Fms), M-CSF could drive the commitment of haematopoietic stem cells to macrophage and osteoclast precursors, principally stimulating the proliferation of osteoclast precursors and enhancing the expressions of RANK (Fig.1-7) (Pereira et al., 2002). Upon maturation, multinucleated osteoclasts have the capacity to release specific molecules such as TRAP and matrix metalloproteinase (MMP) families to demineralize and degrade bone ECM (Mellis et al., 2011; Sommerfeldt and Rubin, 2001). However, the underlying mechanisms by which osteoclast precursors are selectively recruited from the circulation into appropriate stromal sites on bone surfaces and thereafter differentiate into bone-resorbing osteoclasts still remain elusive. The average lifespan of human osteoclasts is approximately 2 weeks (Manolagas, 2000).

1.3 Bone Growth and Development

1.3.1 Overview of Bone Growth and Development

Bone growth development is a process consisting of diverse morphological and biochemical changes during fetal and postnatal development, although peak growth is achieved during the end period of adolescence (van der Eerden et al., 2003). Growth of the human skeleton commences with the aggregation of MSCs into mesenchymal condensations at early stage of embryonic development (Calmar and Vinci, 2002). By the end of the eighth week of gestation, the skeletal pattern is formed in both cartilages and the connective tissues, leading to the commencement of ossification (Iannotti et al., 2000). Ossification is the process of bone formation carried out by two distinct pathways, namely: endochondral and intramembranous

ossification (Kini and Nandeesh, 2012). Endochondral ossification involves a cartilage model as an intermediate (Fig.1-8), while intramembranous ossification is the direct replacement of mesenchymal connective tissue by bone tissue (Fig.1-9) (Karaplis, 2002). The initial location of ossification is termed as the primary ossification centre (Hartmann, 2006). In some bones (e.g. long bones), the primary ossification centres do not develop into the entire region of intermediate so that the secondary ossification centres are formed to cover the reaming regions. These secondary ossification centres commonly develop during postnatal development (Scheuer et al., 2000) and are different in number, location, and time of appearance among various species (Patton and Kaufman, 1995).

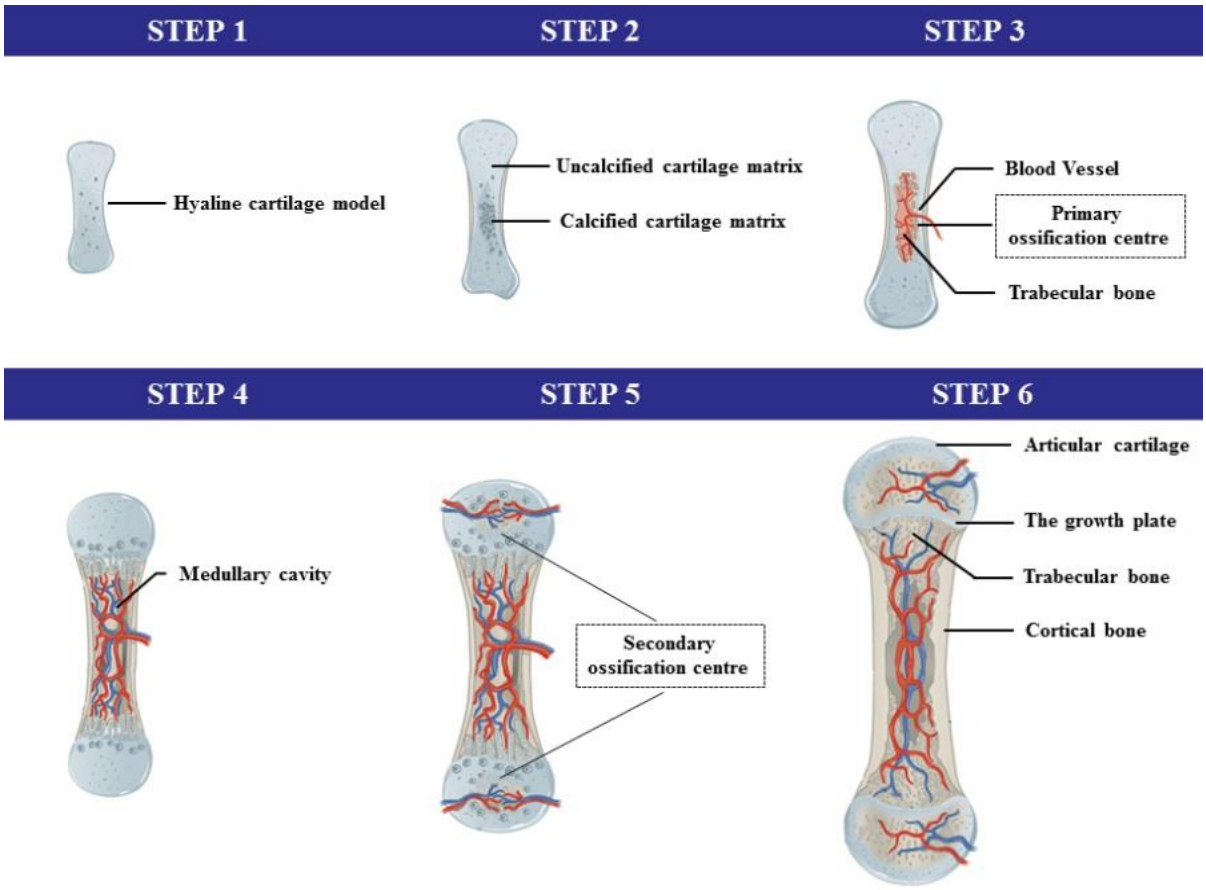


Figure 1-8. Schematic representation of endochondral ossification. 1. Formation of hyaline cartilage model; 2. Development of cartilage model; 3. Development of the primary ossification centre; 4. Further growth in length and diameter; 5. Development of the secondary ossification centres; 6. Formation of the growth plate and articular cartilage. Figure was adapted from (Marieb and Hoehn, 2007).

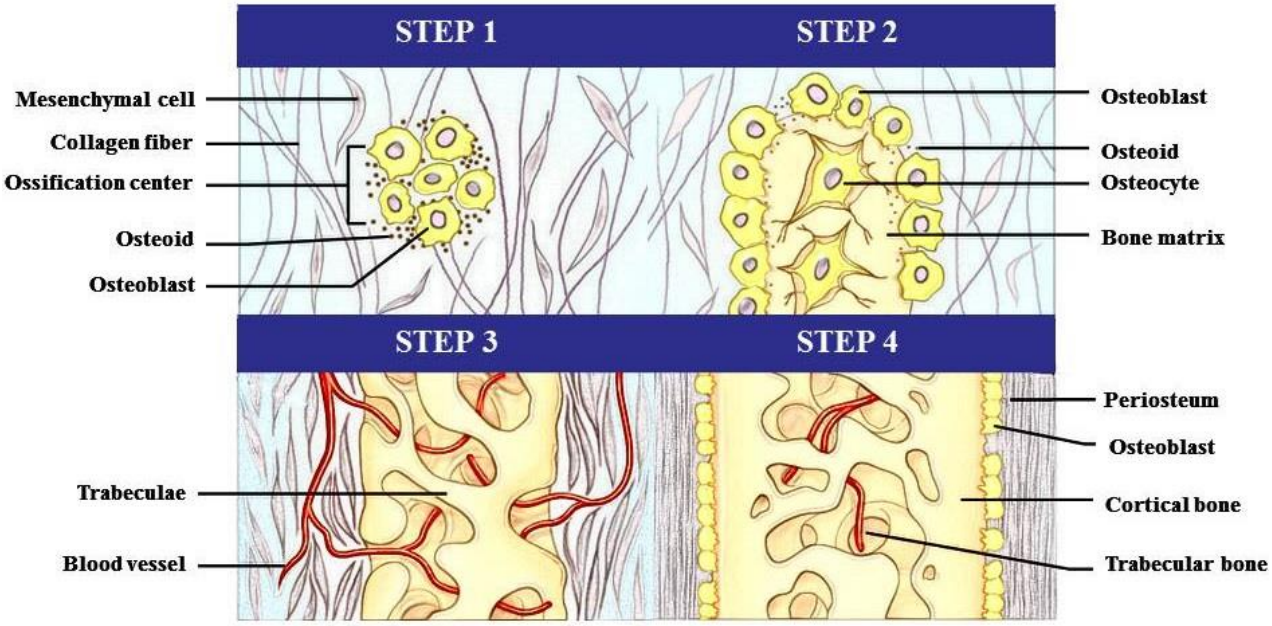


Figure 1-9. Schematic representation of intramembranous ossification. 1. Development of ossification centre; 2. Calcification; 3. Formation of trabecular bone; 4. Formation of periosteum. Figure was adapted from (Hernandez et al., 2004).

During fetal development, long bones form through the process of endochondral ossification, whereas flat bones and less commonly short bones develop by intramembranous ossification (Ralston, 2009). In addition, woven bone (synonymously as primary bone), the immature bone tissue initially formed in either endochondral or intramembranous ossification, is shown to first appear at the fourth month of fetal development, and subsequently mature into a more optimal structure termed lamellar bone (synonymously as secondary bone) just before birth (Teti, 2011). Compared with lamellar bones, woven bones are weaker and lacks of the resistance to torsion forces, due to the absence of concentric lamellaes that make up the osteons (Tortora and Petti, 2002) (Fig.1-10).

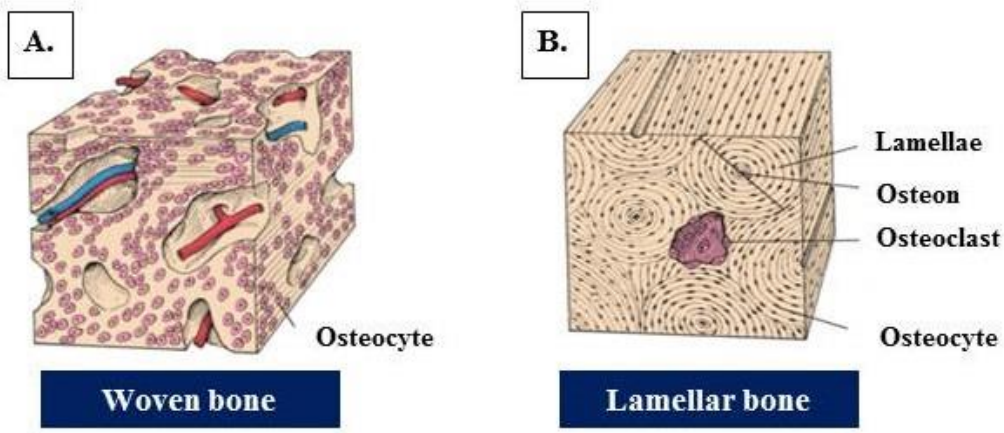


Figure 1-10. Woven bone versus lamellar bone. The collagen fibers are deposited in a disorganized manner in woven bones (A) but in a highly organized concentric manner in lamellar bones (B). Figure was adapted from (Shapiro, 2008).

Bone subsequently undergoes longitudinal and radial growth during postnatal development (Clarke, 2008). Expansion of bone mass is ascribed to endochondral ossification within the growth plates as well as intramembranous ossification on the surfaces of periosteum and endosteum (Prentice et al., 2006). Endochondral ossification ceases during late stage of adolescence and the growth plates eventually fuse when the peak height is achieved, whereas intramembranous ossification slowly continues throughout life. Meanwhile, the outer envelope of bone retains expansion via periosteal apposition during adulthood whilst the endosteal surface expands or shrinks relying on the stages of life (e.g. pregnancy, lactation and menopause) (Prentice et al., 2006). In addition, ossification and bone development also involve two indispensable processes: bone modeling and remodeling (see details below), by which bone is constantly reshaped and renewed throughout life (Ralston, 2009).

1.3.2 Longitudinal Growth of Bone

As stated above, intramembranous ossification is the direct mineralization process of bone

matrix produced by osteoblasts, whereas endochondral ossification is referred as deposition of bone matrix on cartilage model (Kini and Nandeesh, 2012). However, the role of the cartilage model is not only to act as a template for bone formation, but also to serve as the foundation for longitudinal bone growth. In brief, longitudinal growth of bone is achieved by the process of endochondral ossification at the growth plate in childhood and adolescence (Xian, 2007).

The normal growth plate is an organized cartilaginous structure situated at each end of the long bone and formed after the appearance of secondary ossification centers (Zoetis et al., 2003). It is comprised of chondrocytes arranged in columns which are parallel to the long axis of the bone. Chondrocytes are derived from chondrogenic progenitor cells differentiated from MSCs (Fig.1-3). In particular, chondrocytes in the growth plate are observed to locate in distinct and fixed zones while they proceed through the different stages of differentiation (Hunziker et al., 1987). Thus, the growth plate is divided into three horizontal zones with distinctive morphological and biochemical stages during chondrocyte differentiation: the resting zone, the proliferative zone, and the hypertrophic zone (Fig.1-11) (Lui and Baron, 2011).

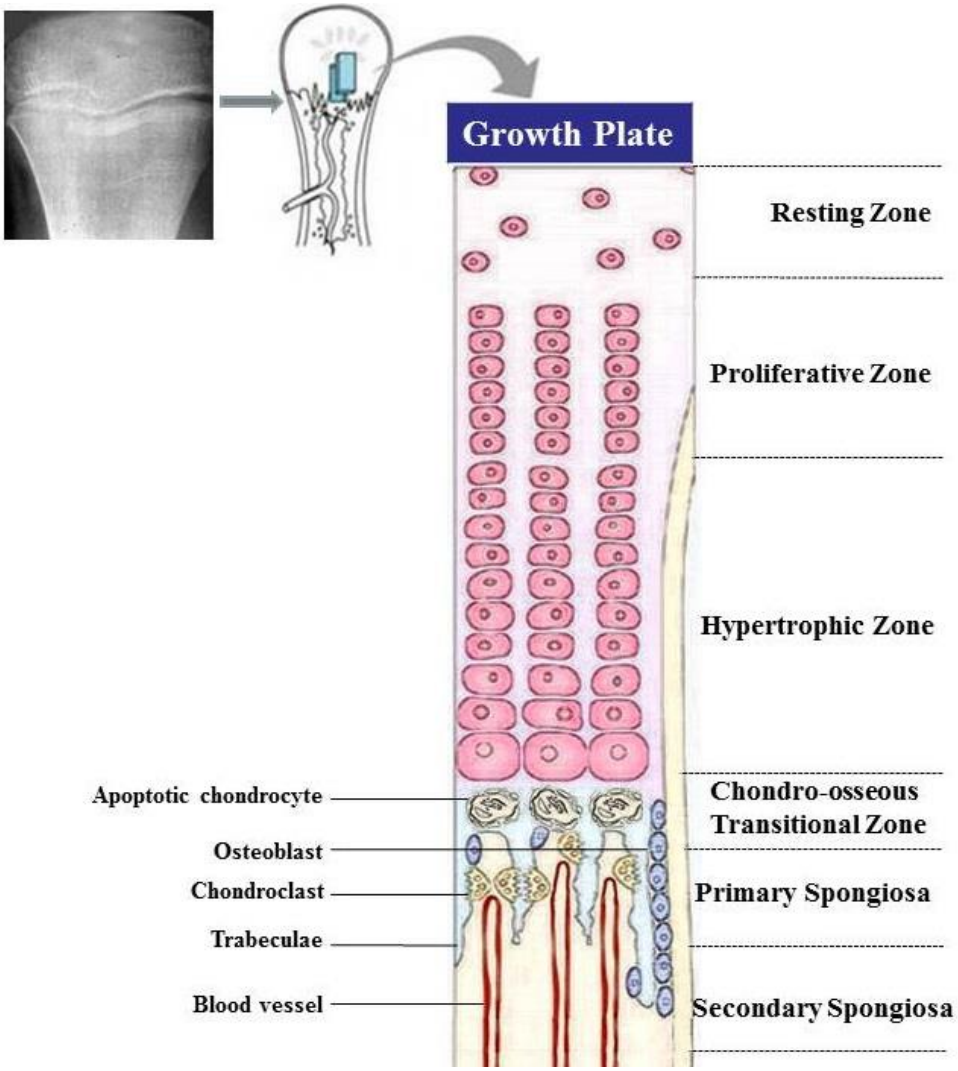


Figure 1-11. Endochondral ossification in the growth plate. The growth plate is a cartilaginous structure situated at the ends of long bones and made up of three distinct zones of unique stages during chondrocyte differentiation, namely the resting, proliferative and hypertrophic zones. Cartilage matrix mineralization progressively follows the apoptosis of hypertrophic chondrocytes, followed by vascular invasion at metaphyseal chondro-osseous transitional zone. Thereby invaded blood vessels enable recruitment of osteoblasts and osteoclasts for bone formation and remodeling on the vascularized calcified cartilage scaffold formed. Figure was adopted from (Xian, 2007).

In addition, chondrocytes are stated to perform highly specialized synthesis and secretion of cartilage matrix into the extracellular space, which provides an environment of nutrition diffusion for chondrocytes as well as endues the joint surface with biomechanical competence (Zuscik et al., 2008). The ECM of cartilage is mainly composed of collagens (e.g. type II, IX, and XI collagens), proteoglycans (e.g. aggrecan) and other non-collagenous proteins (e.g.

cartilage oligomeric matrix protein) (Mackie et al., 2011). As chondrocytes undergo differentiation within the growth plate, the compositions of ECM vary depending on the stage of differentiation and could act as molecular markers for assessing chondrogenesis (Fig.1-12).

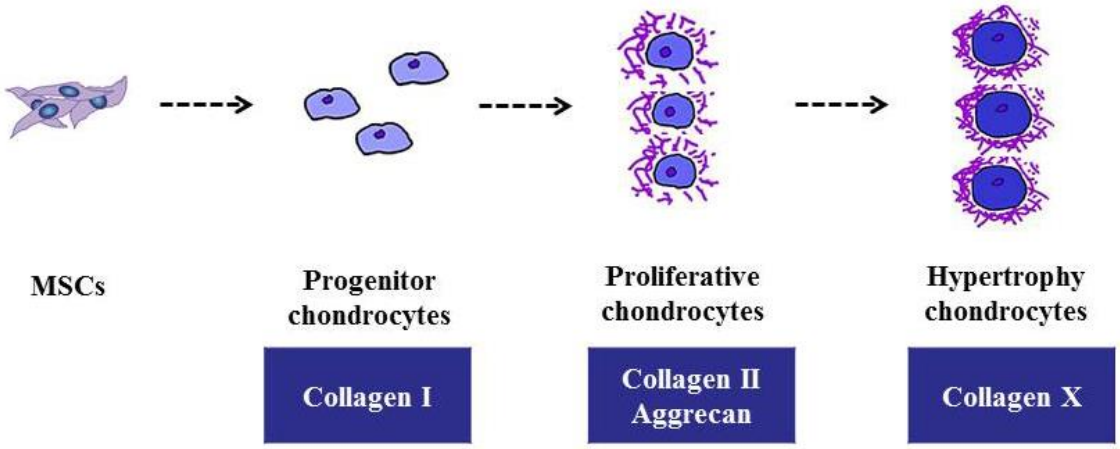


Figure 1-12. Schematic illustration of chondrocyte differentiation and selective molecular markers. The process of chondrocyte differentiation is related to some unique and altered expressions of specific cartilage matrix proteins such as type II and type X collagen. Figure was adapted from (Bonfield and Caplan, 2010).

In the resting zone of the growth plate, progenitor chondrocytes that are capable of forming proliferative chondrocytes are located sporadically at the top of the growth plate. Cells within this zone remain in a relatively quiescent state and exhibit no/or slow division or active matrix production (Ballock and O'Keefe, 2003). These cells have low ionized calcium content and the ratio of ECM to intracellular volume is high (Mackie et al., 2008). ECM synthesized by the undifferentiated progenitor chondrocytes is demonstrated to be rich in type I collagen in spite of low proteoglycan and type II collagen production (Gordon and Hahn, 2010). They are thought to be stored as a nutrient source and be critical for the orientation of columnar alignment of chondrocytes and unidirectional bone growth (van der Eerden et al., 2003).

In the proliferative zone, chondrocytes undergo rapid cell division in which they adopt a

flattened, oblate shape and arrange themselves in organized columns parallel to the long axis of the bone (Ballock and O'Keefe, 2003). It has been reported that eight new chondrocytes are proliferated in each column per day (Farnum et al., 2000). Meanwhile, ECM mainly consisting of collagens (e.g. type II, IX and XI collagens) and proteoglycans (e.g. aggrecan) is highly and actively produced by these cells, which enables separation of the recently divided cells from one another (Mackie et al., 2011). The collagen fibrils provide the framework for the tissue; while the strongly hydrophilic aggrecan acts as a lubricant or a shock absorber of the tissue. Cartilage-specific type II collagen becomes the most abundant collagen species in the growth plate and present in all zones particularly in the proliferative zone (Zuscik et al., 2008).

In the hypertrophic zone, which are located farthest from the end of the bone in these columns, chondrocytes lose their capacity to divide and further progress in the differentiation pathway to become hypertrophic chondrocytes which have a round appearance (van der Eerden et al., 2003). This progress is associated with a significant increase in chondrocyte intracellular volume (five to tenfold increase) as well as unique alterations of ECM (Hunziker, 1994; O'Keefe et al., 1994). In particular, Matrix metalloproteinase (MMP) family (e.g. MMP-13) was found to be implicated in this process, resulting in the degradation of pericellular matrix and the expansion of chondrocytes (Johansson et al., 1997). As chondrocytes reach hypertrophy, the cartilage is characterised by increased expression of type X collagen (O'Keefe et al., 1994) and the reduction of type II collagen (Suda et al., 1999). Compared with type II collagen, non-fibrillar type X collagen is only found in the hypertrophic zone of the growth plate and is more easily resorbed (Poole and Pidoux, 1989). Although the specific

functions of type X collagen remain unclear, it has been revealed that it may act as a calcium-binding protein to facilitate the calcification of the hypertrophic cartilage matrix (Shen, 2005), which indicates the termination of chondrogenesis (Shen et al., 2006). Therefore, type II and X collagens are ideal molecular markers for chondrogenesis (Fig.1-12). While the early phase of chondrocyte differentiation is characterized by inductive expression of the type II collagen gene, the late phase is characterized by the onset of expression of the type X collagen gene (Kartsogiannis and Ng, 2004).

It is evident that the role of terminally differentiated hypertrophic chondrocytes is to mineralize their surrounding matrix, acting as a scaffold for future bone formation (van der Eerden et al., 2003). Hydroxyapatite crystals are deposited surrounding these cells within ECM. Meanwhile, vascular endothelial growth factor (VEGF) is released by hypertrophic chondrocytes to trigger vascular invasion (Mackie et al., 2011). As mineralization of the cartilage matrix makes it impermeable to the diffusion of nutrients, inner chondrocytes begin to undergo apoptosis (Green, 1998), leaving empty lacunae to the vascular system and bone-marrow stromal cells (Roach et al., 1998). Following chondrocyte apoptosis, the calcified cartilage is penetrated by blood vessels from the adjacent primary spongiosa region of the metaphysis (Ballock and O'Keefe, 2003). Vascularization subsequently establishes the channels for highly specialized cells such as osteoclasts and osteoblasts (Mackie et al., 2011). Some proteolytic enzymes such as MMP-13 were expressed by and around forming blood vessels, which is obligatory for cells to penetrate the underlying basal lamina barrier and initiate their migration (Karelina et al., 1995). In addition, osteoclasts identified within this particular region are characterized as chondroclasts (Sims and Johnson, 2012) which are

specifically involved in the resorption of calcified cartilage (Vu et al., 1998). Approximately two-thirds of cartilage matrix is actively degraded by chondroclasts, whilst the cartilaginous remnants acts as a template for bone matrix deposition by osteoblasts to form trabecular bones (Ballock and O'Keefe, 2003). Consequently, primary trabeculae consisting of the spicules containing both mineralized cartilage and bone matrix are formed in the primary spongiosa, and further remodeled into secondary trabeculae in the secondary spongiosa (Kronenberg, 2003). The chondro-osseous transition zone is illustrated to delineate the boundary between the cartilage and the primary ossification center (Fig.1-12) (Tsang et al., 2015).

The net result of endochondral ossification bone growth is that cartilages are progressively synthesized and thereafter replaced by bones at the base of the growth plate, leading to an increase in longitudinal growth. Endochondral ossification ceases during adolescence, by the time the skeletal maturity is achieved (Teti, 2011). As the primary ossification centre meets the secondary centres, the growth plates eventually fuse and the original cartilage model is completely replaced, apart from a thin piece of hyaline cartilage remaining at the articular surface of epiphyses, termed as articular cartilage (Fig.1-8) (Kini and Nandeesh, 2012).

1.3.3 Appositional Growth of Bone

Aside from growing in length, bones also normally increase in diameter to accommodate the increased load during postnatal growth (Parfitt, 1994). Different with longitudinal growth, long bones grow circumferentially by appositional growth, which is a form of intramembranous ossification that involves the osteogenic fibrous connective tissue termed

periosteum, and continue throughout life (Fawcett, 1994). In particular, appositional growth of bone is confirmed to be one of the most important determinants of bone strength and bone stability (Rauch, 2005). During appositional growth, osteoblasts beneath the cellular layer of periosteum are responsible for forming new osteons on the external bone surface. On the other hand, osteoclasts concurrently resorb bone at the endosteal surface (Clarke, 2008). Although the net result is an increase in both the diameter and marrow cavity of the bone, the thickness of cortical bone within the diaphyseal shaft remains fairly constant due to the coupled periosteal deposition and endosteal resorption (Zoetis et al., 2003).

1.3.4 Bone Modeling

Once bone has been formed, it is still not a rigid structure which requires reshaping and rebuilding throughout life (Seeman, 2009). Bone modeling is a process by which bone mass and architecture are adapted in response to physiological impacts or mechanical forces, resulting in gradual adjustment of the skeleton to enable functional requirements (Lin et al., 2006). It principally continues throughout childhood and adolescence until peak bone mass has been achieved (Kini and Nandeesh, 2012). During bone modeling, bones may widen or change axis to sculpt themselves properly by the addition or removal of bone on diverse bone surfaces owing to uncoupled action of osteoblasts and osteoclasts (Fig.1-13) (Teti, 2011).

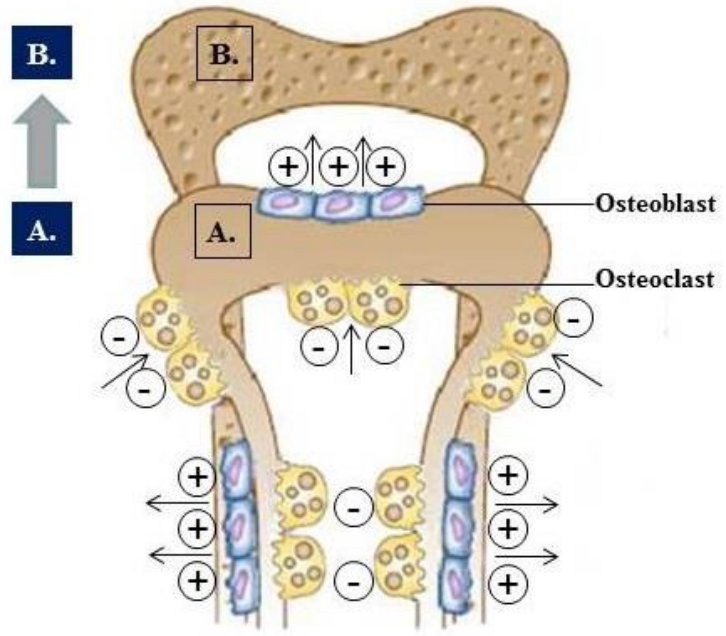


Figure 1-13. Schematic illustration of bone modeling. The sites in which osteoblasts deposit bone matrix and osteoclasts resorb bone matrix are separate and independent during modeling. The uncoupling bone formation (+) and resorption (-) activities alter the long bone shape from A to B, resulting in the increase in length and diameter. Figure was adapted from (Teti, 2011).

1.3.5 Bone Remodeling

Bone remodeling is a process that bone resorption is tightly co-ordinated with bone formation, which is crucial to supply calcium for extracellular function, to repair and replace old bone, and to form new bone to maintain bone mass, bone strength, and skeletal flexibility (Rucci, 2008). Bone remodeling occurs and continues throughout the entirety of life (Xian, 2007). During bone modeling and/or remodeling, bone is resorbed by osteoclasts and new bone is deposited in the resulting cavities by osteoblasts (Fig.1-14) (Rucci, 2008). Although they are seemingly similar, these two processes have distinct mechanisms whereby the orchestration of bone cell activities is uncoupled (in modeling) or coupled (in remodeling) (Teti, 2011). In addition, it is demonstrated that bone remodeling is more frequent than modeling in adults

and approximately 10% of the adult skeletons are being remodeled at any time (Ralston, 2009).

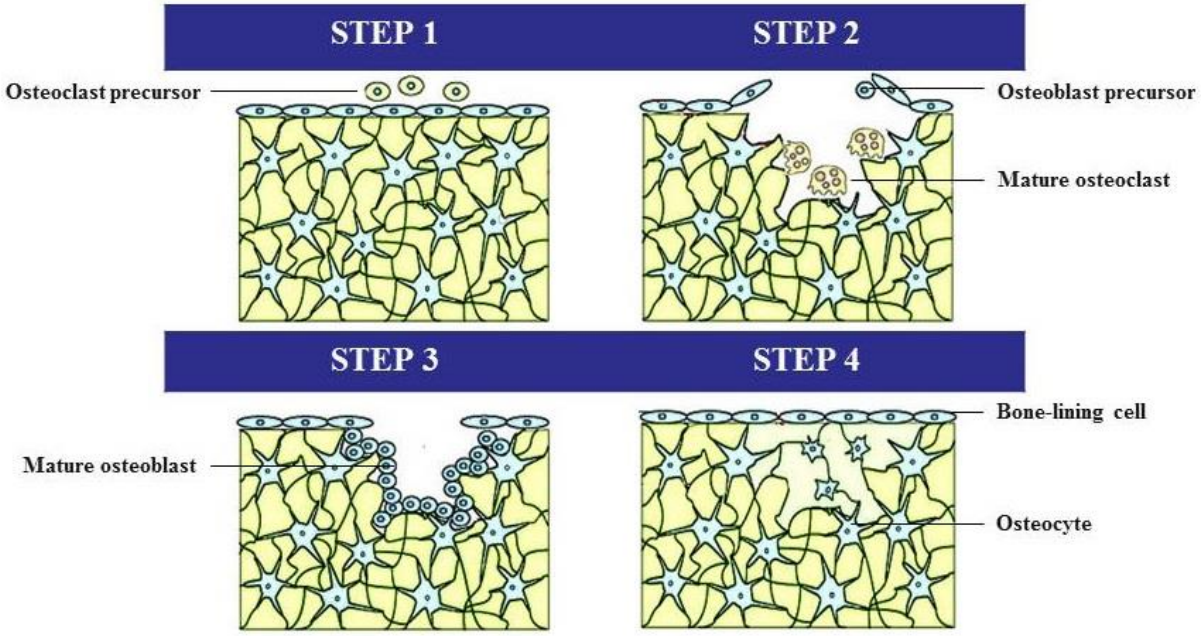


Figure 1-14. Schematic illustration of bone remodeling. 1. Osteoclast precursors attach to bone; 2. Osteoclast precursors differentiate into mature osteoclasts and form a resorption lacuna; 3. Osteoblast precursors are then recruited, followed by proliferating and differentiating into mature osteoblasts. Mature osteoblasts secrete osteoid to resorption lacunae; 4. After the lacuna has been filled, osteoblasts transfer to quiescent bone-lining cells or osteocytes, followed by calcification of bone matrix. Figure was adapted from (Rucci, 2008).

Under physiological circumstances, there is a balance between the coupling bone formation and resorption during bone remodeling (Rosen et al., 2009). This ongoing balance is responsible for the constant but subtle reshaping of bones. New bones will be formed and old or damaged bones will be repaired and replaced at the same site on a bone surface. However, in situations such as glucocorticoid therapy, endochondral ossification will be impaired and bone remodeling will be disrupted to favor bone resorption rather than formation, resulting in secondary bone growth defects and bone loss (Hardy and Cooper, 2009).

1.3.6 Regulation of Bone Growth, Development and Maintenance

The process of bone growth and development is characterized by three phases (van der Eerden et al., 2003): 1. Rapid growth from fetal life to three years of age; 2. Slowly decelerating growth to puberty age; and 3. Increased rate of longitudinal bone growth until the peak height is reached. As afore mentioned, bone is a metabolically active tissue and bone growth and development comprise a complicated coordination among multiple cell categories, however the underlying molecular mechanisms are only partially understood. Aside from genetic as well as life-style and environmental (e.g. physical activity and nutrition status) factors, numerous interrelated regulators have been demonstrated to influence the differentiation and activity of skeletal cells and then regulate the growth and development of bone (Raisz, 1999). In brief, bone growth and development could be systemically controlled by specific factors such as growth hormone (GH), insulin-like growth factor 1 (IGF-1), the thyroid hormones, sex hormones, and glucocorticoids, whereas local factors such as Indian hedgehog (Inh), parathyroid hormone-related peptide (PTHrP), and FGFs also play essential roles in the growth and development of bones (Rauch, 2005).

1.3.6.1 Systemic regulation

Normal bone growth and development are coordinated by the accurate functioning of the endocrine system. GH is synthesized and secreted from the pituitary and primarily exerts its effect to accelerate chondrocyte proliferation within the growth plate during postnatal development, which is mainly dependent on the action of IGF-I produced in the liver upon action of GH (Pass et al., 2009). Although the GH/IGF-I axis is considered as a vital regulator

of longitudinal growth of the bone, previous studies have shown that GH has an IGF-I-independent local action on the growth plate via GH receptors (GHRs) and GH biding proteins (GHBPs) (van der Eerden et al., 2003). Aside from promoting growth plate chondrocyte proliferation, GH exerts both direct and indirect effects on bone formation by stimulating osteoblast proliferation and differentiation, increasing their number and function (Hull and Harvey, 2000).

Thyroid hormones also play a crucial role in bone growth and development. Thyroid hormone triiodothyronine (T3) is demonstrated to be capable of inducing chondrocyte hypertrophy, acted through thyroid hormone receptors (TRs) directly or partly mediated by interference with the GH/IGF-I axis (Mackie et al., 2008). It is evident that T3 and its prohormone, thyroxine (T4), possess two opposing actions during bone development to promote both bone formation and resorption, which is critical to the coordination of normal bone remodeling (Kawaguchi et al., 1994).

Glucocorticoids (GCs) are illustrated to exert direct actions on chondrocytes in the growth plate by binding to the glucocorticoid receptors (GRs), resulting in inhibited chondrocyte proliferation (Annefeld, 1992) and increased chondrocyte apoptosis (Chrysis et al., 2005). GCs are also able to modify the GH-IGF-I axis via inhibiting the secretion of GH, reducing the expression of GHR and IGF-I, as well as altering the synthesis of IGF binding proteins (IGFBPs), leading to the suppression of bone growth (van der Eerden et al., 2003). The activity of deiodinase which is the determinant for the conversion of thyroxine (T4) to T3 is illustrated to be regulated by GCs so that GCs may modulate thyroid local expression in the

growth plate and consequently influence bone growth (Miura et al., 2002). Furthermore, GCs have also been shown to cause other adverse effects during bone development including insufficient ECM synthesis, decreased osteoid mineralization, increased osteoblast and osteocyte apoptosis, and prolonged life span of osteoclasts (see details below), leading to inhibited bone formation and stimulated bone resorption (Hofbauer and Rauner, 2009). Thus, GC is a potent negative regulator of bone growth, development and maintenance, not only through their direct effects but also by interacting with other growth-modulating systemic factors.

Calcium homeostasis is controlled by parathyroid hormone (PTH) through its direct action on the bone and the kidneys as well as the indirect influence on the intestine. PTH has been illustrated to be an active inducement for bone resorption and exerts a biphasic effect on bone formation. Although the synthesis of collagen is acutely inhibited under high concentrations of PTH, bone formation has been shown to be promoted with prolonged intermittent administration (Datta and Abou-Samra, 2009). Previous studies have shown that calcitriol can stimulate bone mineralization by supporting the intestinal absorption of calcium and phosphate. On the other hand, it has been demonstrated to be a vital local regulator of osteoclast differentiation (Raisz, 1993). Moreover, calcitonin (CT) is shown to act as an inhibitor of bone resorption which has the capacity to reduce the number and activity of osteoclasts as well as increase the excretion of calcium and phosphorus (Renkema et al., 2008).

Sex steroids such as estrogens, progesterone and androgens are known as essential regulators

for bone development and maintenance, especially during puberty (van der Eerden et al., 2003). Estrogens have been shown to exert dual effects on bone metabolism. They have the capacity to coordinate local regulators of osteoblastogenesis and increase the levels of OPG, resulting in impaired bone formation and reduced bone resorption. However, the inhibition effect to bone formation is less significant than that to resorption, thus bone mass could be ultimately increased (Raisz, 1999). Both progesterone and androgens have the anabolic effects on bone through stimulating their osteoblast receptors (Kini and Nandeesh, 2012). It has been revealed that approximately 30% females develop osteoporosis (type-I) after menopause due to estrogen deficiency (Pietschmann et al., 2009). Decrease in estrogen level is found to stimulate the expression of interleukin (IL)-6 and other cytokines which accordingly results in enhanced osteoclast recruitment and activation of osteoclasts, as well as increase in the sensitivity of bone resorption caused by the promoting action of parathyroid hormones (Khosla et al., 2011). In addition, age-related osteoporosis (type-I) is illustrated to be the predominant form of osteoporosis in people over the age of 70 years and twice as frequent in females as in males, which is also associated with estrogen deficiency (Pietschmann et al., 2009).

1.3.6.2 Local regulation and maintenance

Aside from systemic regulators, locally produced and secreted factors, among which are principally growth factors, morphogens and cytokines, also play a critical role in regulating bone growth, development and maintenance (Raisz, 1999). Growth factors are polypeptides acting as modulators of the cellular proliferation, differentiation and function, among which the most abundant in the bone matrix are IGFs (Govoni, 2012). IGFs have been confirmed to

increase the number and function of the osteoblasts as well as stimulate synthesis of collagens (Delany et al., 1994). As discussed above, IGFs could also circulate while linked to associated IGFBPs, which in turn stimulates or inhibits IGF action to regulate local bone remodeling (Conover, 2008).

Transforming growth factor beta (TGF-β) is the second most abundant growth factor in bone tissue and is a potent inducer of bone formation. It has been demonstrated that TGF-β plays an essential role in condensation, proliferation, differentiation of growth plate chondrocytes during endochondral ossification, particularly acts as a compelling inhibitor for chondrocyte terminal differentiation (Wang et al., 2014). TGF-β has been also shown to be capable of promoting osteoblast differentiation and osteoid synthesis as well as inhibiting synthesis of some proteases such MMPs (Chen et al., 2012). On the other hand, it is also evident that TGF-β could inhibit bone resorption by suppressing osteoclast formation, differentiation and activity as well as inducing their apoptosis (Pivonka et al., 2008).

Bone morphogenetic proteins (BMPs) are also the members of the TGF-β superfamily (Guo and Wang, 2009) and have been shown to play multiple roles in skeletal development and maintenance (Canalis et al., 2003). It has been shown that the BMP family has the capacity to enhance chondrogenesis during various stages, from initial commitment to terminal differentiation (Yoon and Lyons, 2004). In particular, some specific BMPs (e.g. BMP-2) have been shown to have the potential to promote chondrocyte proliferation and hypertrophy within growth plate, leading to an accelerated longitudinal bone growth (De Luca et al., 2001). However, these promoting effects of BMPs on chondrogenesis could be antagonized by FGF

signaling (Minina et al., 2002). FGFs are essential regulator of chondrocyte proliferation via binding to FGF receptor 3 (FGFR3), while the activation of FGFR3 could cause a significant suppression in chondrocyte proliferation (Du et al., 2012). Therefore, the interaction of BMP and FGF signaling could coordinate the differentiation process of chondrocytes. In addition, it has been illustrated that BMPs are also a potent inducer of bone formation through its stimulating effects on osteoblastogenesis (Wu et al., 2016). Some specific BMPs (e.g. BMP-2) are demonstrated to regulate the expression of Runt-related transcription factor 2 (Runx2) and Osterix (Osx) to stimulate osteoblast differentiation (Lee et al., 2003a; Lee et al., 2003b; Miyama et al., 1999). Runx2 is the dominant osteoblast transcription factor which promotes the early stages of MSCs commitment towards osteoprogenitor cells (Karsenty and Wagner, 2002; Olsen et al., 2000), preserves osteogenic cells in an immature stage and prevents osteoblast terminal differentiation (Komori, 2006). Osx acts downstream of Runx2 and also exerts considerable influence on osteoblastogenesis by promoting pre-osteoblasts to become immature osteoblasts (Kobayashi and Kronenberg, 2005).

Parathyroid hormone-related protein (PTHrP) is mainly expressed by early proliferative chondrocytes and shares a common receptor with PTH (Juppner et al., 1991). PTHrP is illustrated to sustain the chondrocytes in the proliferative stage and prevent them from entering hypertrophy maturation (Lee et al., 1996). The suppression of hypertrophy is partly through the inhibition of Runx2 expression (Guo et al., 2006) and also indirectly mediated by chondrogenic transcription factor SOX9 which interferes with the expression Runx2 to delay hypertrophy (Yamashita et al., 2009).

Indian hedgehog homolog (Ihh) is a member of hedgehog protein family, which has the capacity to stimulate chondrocyte proliferation and inhibit chondrocyte hypertrophy via promoting PTHrP expression (Mackie et al., 2008). The interactions of Ihh and PTHrP may establish a feedback loop to retain chondrocytes in the proliferative pool (Kronenberg, 2003). Ihh has also been shown to play an indispensable role in osteoblast development (Long et al., 2004). It has been demonstrated that Ihh exerts direct effects on osteoprogenitor cell commitment and the expression of Runx2 could not be activated in the absence of Ihh (St-Jacques et al., 1999). Therefore, Ihh is identified as a coordinator between osteogenesis and chondrogenesis (St-Jacques et al., 1999).

It is evident that Wnt (a portmanteau of Wingless and integration) family act as downstream factors of Ihh to regulate osteoblast differentiation (Hu et al., 2005). Wnts are secreted glycoproteins which are essential for skeletal cell determination, proliferation and development (Brennan-Speranza et al., 2012; Caetano-Lopes et al., 2006). Some Wnt proteins (e.g. Wnt3a and Wnt10b) are capable of stimulating Runx2 expression by activating canonical Wnt/β-catenin signaling (Fakhry et al., 2013), which is an enticing pathway implicated in bone development, particularly in osteoblast differentiation (Fig.1-15) (Hardy and Cooper, 2009). In this complex signaling pathway, Wnt proteins bind to the receptor frizzled (Fz) and its co-receptor low-density lipoprotein receptor-related protein 5/6 (LRP5/6), which in turn transduces Wnt signals to stabilize transcription factor β-catenin. It results in the transcription of osteoblast-specific genes which regulate bone formation (Shoback, 2007). In addition, canonical Wnt/β-catenin signalling pathway is found to be promoted by the destabilization of the intracellular degradation complex involving glycogen synthase kinase-3β (GSK-3β). This

pathway could also be inhibited by various antagonists including secreted proteins such as secreted frizzled related protein-1 (SFRP-1), dickkopf-1 (Dkk-1) and sclerostin (encoded by SOST gene in osteocytes), which would prevent Wnt binding to its receptor complex (Canalis et al., 2007). Besides being important for promoting osteogenesis, specific Wnt proteins, particularly Wnt10b, have been shown to inhibit adipogenesis in the bone marrow microenvironment by depressing the expression of crucial adipogenic transcription factors such as peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα). Therefore, any abatement of Wnt/β-catenin signaling pathway may lead to a disruption of the balance between osteogenesis and adipogenesis, resulting in the following bone growth defects. In addition, Wnt signaling is also shown to play an essential role in osteoclast formation (Macsai et al., 2008). Collectively, Wnt signaling is a crucial regulator of chondrogenesis, osteoblastogenesis, osteoclastogenesis, endochondral bone formation, and bone remodeling throughout life.

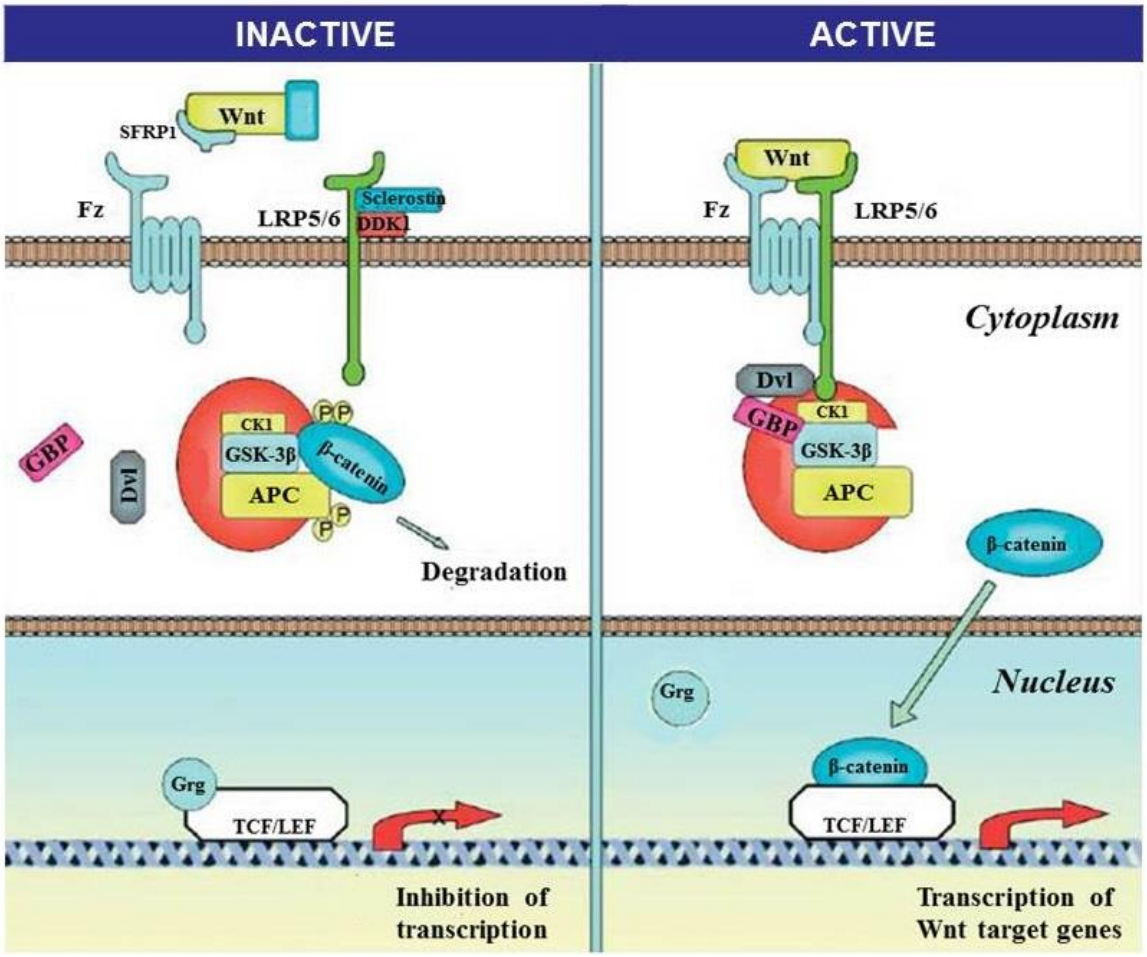


Figure 1-15. Canonical Wnt/β-catenin signaling pathway. A destruction complex which consists of glycogen synthase kinase-3β (GSK-3β), casein kinase 1(CK1), axin, and adenomatous polypsis coli (APC) targets β-catenin for proteosomal degradation in the absence of Wnt signaling. After binding of Wnts to the frizzled (Fz) and LRP5/6 co-receptor complex, Wnt signaling is activated. Activation causes recruitment of GSK-3β binding protein (GBP), which induces the stabilization of β-catenin. β-catenin accumulates within the cytosol and translocates into the nucleus where it complexes with members of the TCF/LEF family of transcription factors to mediate transcriptional induction of Wnt target genes to promote osteoblast differentiation. β-catenin is then exported from the nucleus and degraded via the proteosomal machinery. Figure was adopted from (Hardy and Cooper, 2009).

Aside from well-established RANKL, it has been stated that some other cytokines, such as TNF-α, IL-1, IL-6, IL-11 and PGs, have both autocrine and paracrine effects in bone development, leading to inflammatory bone loss (Raisz, 1999). Previous studies have shown the upregulation of these systemic inflammatory cytokines is associated with a direct increase in RANKL expression, suggesting these cytokines are profound regulators of the NF-κB

pathway and osteoclast activity, resulting in aggravated bone resorption (Jilka, 2003; King et al., 2012). In addition, it has been found the expression of M-CSF, which is important for proliferation of osteoclast precursor cells, could be regulated by TNF-α to further promote the development of osteoclasts (Besse et al., 1999). Furthermore, members of epidermal growth factor (EGF) family, have been observed to play a role to regulate bone growth and maintenance in endocrine/autocrine/paracrine manners (Xian, 2007).

1.3.6.3 Role of chemokines in bone development and maintenance

Accumulating evidence has revealed their overlapping regulatory mechanisms by which skeletal and immune cells shared the regulators in common, including specific chemokine(s) (Caetano-Lopes et al., 2009). Chemokines are a large family of small chemotactic cytokines and well-known to regulate the migration and positioning of immune cells (Su et al., 2017b). Recently, aside from these systemic and/or local regulators described above, a subset of chemokines have been shown to participate in multiple skeletal cell regulation pathways and play a crucial role in regulating bone growth, development and maintenance under physiological and pathological circumstances (Gilchrist and Stern, 2015). Chemokines are a superfamily consisting of a large number of small chemotactic cytokines which serve as key signals and exert their biological effects through specific membrane receptors (Fig.1-16) (Zlotnik et al., 2006). Based on their structures, chemokines are subdivided into four subfamilies, two major (CC and CXC) and two minor (CX3C and C) subfamilies (Fig.1-16) (Bonecchi et al., 2009).

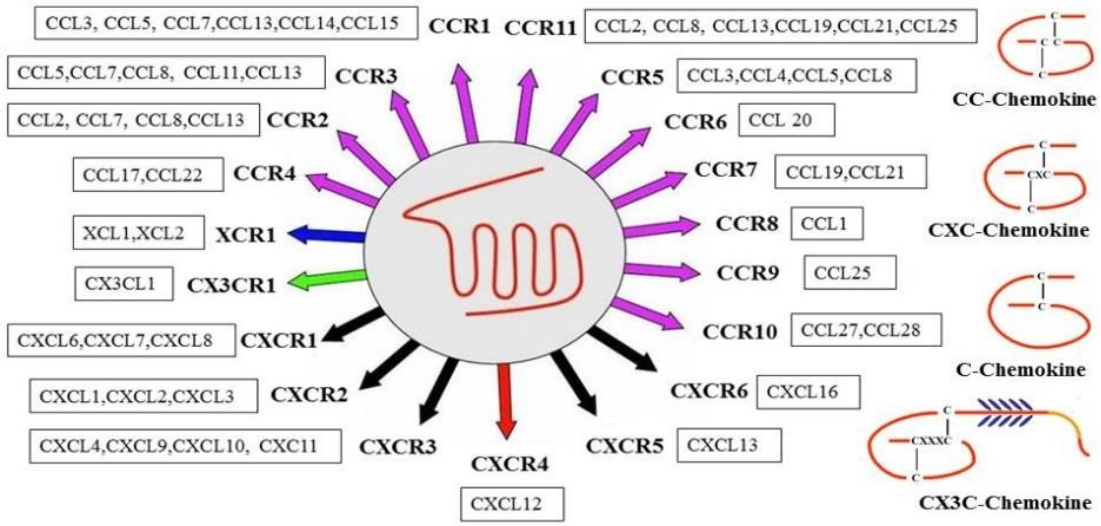


Figure 1-16. Chemokines and chemokine receptors. Chemokines are subdivided into four subfamilies, two major (CC and CXC) and two minor (CX3C and C) subfamilies. In addition, several chemokines have different receptors, and many receptors bind to various ligands. Figure was adopted from (Schall and Proudfoot, 2011).

Aside from their major roles in regulating immune responses and inflammation, chemokines play crucial roles in coordination of trafficking, differentiation, and activity of skeletal cells during bone growth and maintenance (Jung et al., 2006; Murata et al., 2012; Wright et al., 2005; Yu et al., 2003a; Yu et al., 2003b). It is demonstrated that a number of chemokines (e.g. CCL2, CCL3, CCL4, CCL5, CXCL8, CXCL9 and CXCL12) are capable of recruiting osteoclast precursors or stimulating osteoclast formation (Binder et al., 2009; Shahnazari et al., 2013; Silva et al., 2007; Wright et al., 2005; Yano et al., 2005). The expression levels of these chemokines are observed elevated under conditions of induced osteocyte apoptosis, increased osteoclastogenesis and bone resorption (Maruotti et al., 2011; Zernecke et al., 2009). It has also been illustrated that some chemokines (e.g. CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL12) are upregulated by chondrocytes under pathological circumstances (Pulsatelli et al., 1999). In addition, previous studies have shown that several chemokines such as CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL12 are implicated in chondrocyte differentiation and

apoptosis (Pulsatelli et al., 1999; Wei et al., 2010; Wei et al., 2006a), illustrating that chemokines are significant regulatory molecules involved in multiple aspects of bone growth and maintenance.

In particular, CXC chemokine CXCL12 and its unique chemokine receptor CXCR4 have gradually become known to be important in skeletal homeostasis. Recent studies have shown that CXCL12 is capable of eliciting deleterious effects in the cartilage under a pathological circumstance leading to stimulated chondrocyte hypertrophy and cartilage degradation (Wei et al., 2010). It has also been revealed that CXCL12 and its receptor CXCR4 are expressed in a complementary pattern in the growth plate and the cell death of chondrocytes is accompanied by high concentration of CXCL12 at the chondro-osseous transitional zone of the growth plate (Wei et al., 2006b). In addition, aside from RANKL, the well-established cytokine associated with osteoclastogenesis, accumulating evidence demonstrated CXCL12 to play a complementary role in osteoclast precursor cell recruitment and development (Gronthos and Zannettino, 2007). It has been illustrated that CXCL12 has the potential to markedly stimulate the chemotactic recruitment and migration of circulating osteoclast precursors. It does so partly via upregulation of MMP-9, which is an enzyme required for cells to penetrate the underlying basal lamina barrier in the process of their migration to the developing medullary cavity (Engsig et al., 2000). CXCL12 has also been shown to exert a direct effect on osteoclast differentiation, particularly during the early stage, via elevation of precursor cell number as well as promotion of multinuclear cell fusion (Wright et al., 2005). Thus, CXCL12 is able to govern numerous developmental, homeostatic, and pathological processes during bone growth and maintenance.

1.4 Cancer Chemotherapy-induced Bone Growth Defects in Childhood

Bone growth and homeostasis can be influenced by medical treatments including cancer chemotherapy. In 2014, 10,450 new cases and 1350 cancer deaths were reported among children under the age of 15 in the United States (Ward et al., 2014). As a result of the widespread introduction of chemotherapy, a significant and ongoing improvement in survival for childhood cancer patients has been observed (Baade et al., 2010). It is estimated that, dependent on the cancer types, about 80% childhood cancer patients are currently cured with chemotherapy, compared to about only 30% of patients that would live without chemotherapy (Gajjar et al., 2006). Thus, chemotherapy is becoming a mainstay in childhood cancer treatment. Though it has achieved great success in treating paediatric malignancies, both clinical and experimental investigations have now demonstrated that bone growth and homeostasis are strongly influenced by medical treatments particularly by chemotherapy. However, the underlying cellular and molecular mechanisms by which cancer chemotherapy induces bone defects in childhood still remain largely unknown.

1.4.1 Clinical Uses and Side Effects of Chemotherapy

Since the introduction in the 1940s, chemotherapeutic agents have been widely used for the management of malignant disease (Brighton et al., 2005). The clinical uses for chemotherapy are determined by tumor characteristics and individual condition (English, 2010). It is becoming increasingly clear that there is an extensive range of sensitivities to chemotherapeutic agents among different tumor types. In addition, even the same tumor type

observed histologically may differ in diverse patterns of chemosensitivity and performance (Caley and Jones, 2012). Therefore, current chemotherapeutic regimens in clinical practice mostly consist of several agents from different classes in combination, and repeated, regular treatment cycles (English, 2010).

While increasing success including obviously improved survival rate has been made in paediatric oncology by intensifying use of chemotherapy, a significant increase in number of cancer patients and/or survivors has also been reported suffering from multiple treatment complications (Caley and Jones, 2012). The diverse side effects of chemotherapy are illustrated to be principally according to the specific agent, dose, route and schedule of administration and any predisposing patient factors (English, 2010). Although patients in paediatric group usually have a better capacity to endure the acute side effects of cytostatic chemotherapeutic agents, the developing children are shown to be more influenced by the long-term effects of chemotherapy (Bleyer, 1985; te Winkel et al., 2014). In particular, recent studies have illustrated that chemotherapy-induced bone growth defects in childhood such as bone loss, growth arrest, osteopenia and fractures during and/or after chemotherapy are one of the most significant complications that occur in a high prevalence, which may persist into adulthood (Fan et al., 2011; te Winkel et al., 2014). For example, acute lymphoblastic leukemia (ALL), the most common childhood malignancy, has an over 80% of survival rate following chemotherapy involving methotrexate (MTX) and dexamethasone (Dex) (Mitchell et al., 2009). However, children with ALL who received intensive use of MTX and Dex have been demonstrated to develop a lower bone turnover and lower bone mineral density (BMD), as well as achieve a considerable decrease in height velocity during chemotherapy treatment

(Viana and Vilela, 2008).

1.4.2 Cellular and Molecular Mechanisms of Childhood Cancer Chemotherapy-induced Bone Growth Defects

As stated above, bone growth during childhood and adolescence is a dynamic process involving diverse steps such as the production of calcified cartilage in the growth plate, its transformation into woven spongy bone in the primary spongiosa, and further modeling/remodeling into lamellar trabecular bone in the secondary spongiosa (Xian, 2007). These processes are all dependent on the regulation of proliferation, differentiation, activity, and apoptosis of skeletal cells including chondrocytes, osteoclasts, osteoblasts, and osteocytes. Due to this complexity, disruption to steady-state regulation of these cells by chemotherapeutic treatment may contribute to bone growth defects during or after chemotherapy (Fan et al., 2012a; Su et al., 2017a). However, the mechanisms of chemotherapy-induced bone growth defects are complicated and remain unclear so far. It is beyond the scope of this project to detail all types of chemotherapeutic agents and regimens., the current review is focusing on the underlying cellular and molecular mechanisms of bone growth defects caused by MTX and Dex, the two most commonly and intensively used agents in childhood oncology, particularly in the treatment of ALL (Crofton et al., 2000).

1.4.2.1 MTX-induced bone growth defects

MTX is an anti-metabolite that inhibits the enzyme dihydrofolate reductase, blocking thymidylate and purine synthesis (Minaur et al., 2002). It is commonly prescribed at

high-doses (1000–12,000mg/m^2/day) for paediatric malignancy treatment (particularly for ALL) (Minaur et al., 2002) and at lower doses (5–25mg/week) for inflammatory disease treatment (e.g. rheumatoid arthritis) (Cronstein, 2005). It has been shown that lower-dose or less frequent administration of MTX, at a similar dosage and schedule used for rheumatoid arthritis, does not cause significant adverse effects on bone metabolism (Buckley et al., 1997; Fan et al., 2009; Ide et al., 1999); however, long-term intensive MTX treatment has been confirmed to induce severe defects to bone growth in paediatric patients (Ecklund et al., 1997; Mandel et al., 2004; Stanisavljevic and Babcock, 1977) and in growing rats (Fan et al., 2012a).

MTX-induced effects on growth plate

Previous studies have revealed that the damaging effect of MTX on the growth plate is dose-dependent (Fan et al., 2009). Long-term or high-dose MTX chemotherapy has been observed to have the capacity to suppress chondrocyte proliferation and collagen-II synthesis (Xian et al., 2008), as well as induce chondrocyte apoptosis in proliferative and hypertrophic zones where apoptosis does not normally occur (Fan et al., 2011; Xian et al., 2007), resulting in a significant reduction in growth plate thickness. As longitudinal bone growth is achieved by endochondral ossification at the growth plate, where chondrocyte number and growth plate thickness positively correlate with bone elongation. Longitudinal growth of bone may be suppressed following intense MTX chemotherapy. In addition, it has been shown that intense MTX treatment could potentially induce chondroclast recruitment and formation at the site of vascular invasion within chondro-osseous transitional zone (Fan et al., 2012a). This may result in increased resorption of mineralized cartilage, leading to inhibition of calcified

growth plate cartilage feeding into the metaphysis, negatively affecting bone deposition and overall bone volume. Collectively, intense MTX chemotherapy may cause dysfunction of growth plate, resulting in a suppression of longitudinal growth and bone mass accumulation.

MTX-induced effects on osteogenesis

During normal endochondral ossification, newly formed primary spongiosa which is adjacent to the growth plate is further remodeled to secondary spongiosa by osteoblasts and osteoclasts, during which woven bones are replaced by lamellar bones (Xian, 2007). Previous studies have shown that MTX chemotherapy causes direct inhibition of proliferation of bone marrow stromal cells and osteoprogenitor cells (Fan et al., 2009; Xian et al., 2008), and has moderate negative effects on proliferation of preosteoblasts and osteoblasts (Scheven et al., 1995; Xian et al., 2008; Xian et al., 2007), which can directly reduce osteoblastic cell density along the trabecular bone surface, leading to the disruption of endochondral ossification. In addition, despite the lack of obvious effects on osteoblast apoptosis along the trabecular surface, MTX treatment has been shown to significantly induce osteocyte apoptosis, with apoptotic osteocytes found in close proximity to increased osteoclast presence along the trabecular surface, suggesting that osteocyte apoptosis may be related to the increased recruitment of osteoclasts (Shandala et al., 2012). Moreover, it has been demonstrated that bone (re)modeling could be disturbed by the decreased activation of the Wnt/β-catenin signaling pathway following MTX treatment, causing a reduction of osteogenesis in metaphysis and bone marrow (Georgiou et al., 2012b). Intensive MTX treatment has also been revealed to significantly inhibit the formation and mineralization of new osseous matrix, resulting in the impaired process of bone remodeling (Cegiela et al., 2005).

MTX-induced effects on osteoclastogenesis

Previous studies have revealed that MTX chemotherapy can result in an increase in the osteoclast precursor pool, promoting osteoclast formation within the bone marrow microenvironment, as well as enhancing osteoclast density on bone surface (Fan et al., 2009; King et al., 2012; Raghu Nadhanan et al., 2014). Previous studies using a rat model with MTX treatment illustrated an increase in the osteoclast precursor cell pool which positively expressed CD11b, as well as increased osteoclast formation (Fan et al., 2009). Surface marker Mac-1 (CD11b/CD18) which is stimulated by RANKL is crucial for the differentiation of osteoclast precursors into mature osteoclasts (Hayashi et al., 2008). This suggests that chemotherapeutic treatment with MTX may influence osteoclast differentiation at the precursor level. Although the underlying mechanisms for chemotherapy induced-osteoclast formation still remains unclear, it has been established that aside from RANKL, osteoclast differentiation promoted by MTX may likely be due to the pro-osteoclastogenic environment created by chemotherapy treatment in the bone and circulation. More specifically, elevated systemic circulating and/or local levels of IL-1, IL-6, and TNF-α and activation of NF-κB were observed in rats treated with MTX, which might be inductive to osteoclastogenesis (King et al., 2012). However, further studies are required to investigate molecules and action mechanisms involved in the enhanced recruitment and differentiation of osteoclasts following MTX chemotherapy.

MTX-induced marrow adipogenesis

As discussed above, MSCs are pluripotent progenitor cells and are capable of differentiating into various lineages, including osteogenic and adipogenic lineages. The lineage-specific

differentiation is coordinated by various transcription factors, such as PPARγ2 for adipogenesis as well as Runx2 and Osx for osteogenesis (Georgiou et al., 2012). Since adipocytes and osteoblasts share a common precursor, MSCs (Fig.1-3), there may be a reciprocal switch in bone versus fat volume within the bone marrow (Bonfield and Caplan, 2010). Previous studies have found that intensive MTX chemotherapy can cause a significant switch in MSC commitment from osteogenesis to adipogenesis (Georgiou et al., 2012), with up-regulated expression of PPARγ2 and fatty acid binding protein 4 (FABP4) which is an adipocyte-specific protein participating in regulation of PPARγ pathway and essential for the maintenance of lipid metabolism (Makowski et al., 2005), as well as down-regulated expression of Runx2 and Osx (Fig.1-17).

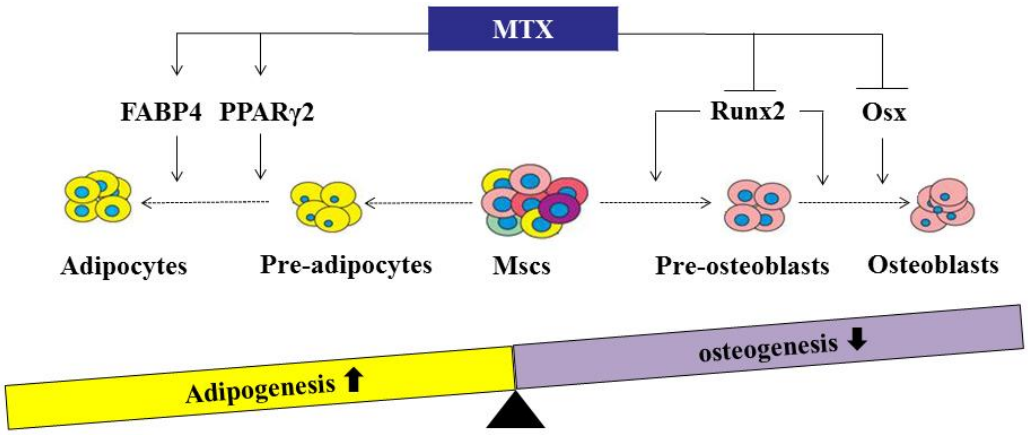


Figure 1-17. MTX-induced bone loss and bone marrow adiposity. MTX chemotherapy stimulates the expression of PPARγ2 and FABP4 but inhibits expression of Runx2 and Osx, resulting increased marrow adiposity and reduced bone volume. Lines with arrowheads indicate stimulatory pathways, and lines with bars indicate inhibitory pathways. Figure was adapted from (Wagner et al., 2010).

1.4.2.2 Dex-induced bone growth defects

Dex is a potent member of the glucocorticoid class of steroids introduced by Dr Philip Hench in the 1950s. Since then it has revolutionized a series of medical treatments including treatments of neoplastic, autoimmune, chronic inflammatory, collagen-vascular, and

granulomatous diseases (Lloyd, 2002). The initial dose of 0.75 - 9 mg/kg/day may vary depending on the specific disease entity being treated and the following response of the patient (Cronstein, 2005). It has been revealed that intensive Dex therapy induce severe adverse effects on the skeleton in the long term (Canalis et al., 2007).

Dex-induced adverse effects on growth plate

Previous studies have shown that when Dex levels rise above physiological concentration, longitudinal bone growth is potently inhibited (Lui and Baron, 2011). The exact molecular mechanisms behind these effects remain largely unknown, however, it has been suggested that growth plate dysfunction and consequent bone growth defects induced by Dex therapy are attributed to suppression of chondrocyte differentiation, proliferation, hypertrophy, increased chondrocyte apoptosis and altered matrix synthesis (Mushtaq et al., 2004; Zaman et al., 2012). Although endocrine signals may be involved to indirectly mediate these effects, recent *in vivo* and *in vitro* studies have confirmed that Dex has direct, local effects that govern growth plate function (Zaman, 2010). *In vivo*, it has been illustrated that the administration of Dex may inhibit chondrocyte differentiation at the early stage of chondrogenesis (Fujita et al., 2004). Direct infusion of Dex to the rabbit tibial proximal growth plate has also been observed to hinder the proliferative and hypertrophy capacity of growth plate chondrocytes, leading to a 77% inhibition in growth compared with the contralateral, vehicle-treated growth plates (Baron et al., 1992). Similarly, *in vitro*, addition of Dex to the culture medium of rat fetal and postnatal metatarsal bones, caused a severe inhibition of growth suggesting a suppressive effect of Dex on chondrocyte proliferation (Chagin et al., 2010). Although increased apoptosis of chondrocytes has been detected in all three zones of growth plate, Dex

is found to stimulate apoptosis principally in terminally differentiated hypertrophic chondrocytes and induce bone growth retardation (Chrysis et al., 2003; Zaman et al., 2012). It has been shown that Dex-induced chondrocyte apoptosis is associated with thinning of the growth plate and bone growth impairment (Zaman et al., 2012). In addition, the reduction in the number of chondrocytes in the growth plate can also suppress type II collagen production, which results in further undesired apoptosis. Furthermore, it has been revealed that cartilage ECM lacking type II collagen cannot support the survival of chondrocytes (Yang, Li et al. 1997). Previous studies in primary cell culture systems suggest a stimulatory effect on proteoglycan synthesis at low Dex concentration but a suppressive effect at a high concentration (Kato and Gospodarowicz, 1985).

Dex-induced effects on osteogenesis

Previous studies have demonstrated that prolonged use of Dex has the potential to induce a reduction in the number of osteoblasts and their capacity to synthesize bone matrix (Bouvard et al., 2010). While there are a number of ways in which Dex may induce such osteogenic defects, studies have found associated altered regulation of both Wnt and BMP signaling in the bone. Both *in vivo* and *in vitro* studies have illustrated that Wnt/β-catenin signaling may be deregulated by Dex administration through up-regulated expression of Wnt antagonists such as Dkk-1 and SFRP-1 as well as down-regulated expression of Wnt receptor complex components Fz-4 and Fz-7 and downstream transcription factor LEF-1, which result in the suppression of β-catenin signaling, leading to the ultimate inhibition of osteoblast differentiation (Bouvard et al., 2010; Georgiou et al., 2012). In addition, previous studies have

confirmed that some bone-anabolic factors such as BMPs may be suppressed by excess Dex (Canalis et al., 2007). In particular, it has been observed that the inhibitory effects of Dex treatment on osteoblast differentiation and mineral deposition could be significantly restored by the administration of exogenous rhBMP-2 *in vitro*, suggesting the induced inhibition of BMP-2 appears to play a central role in the overall adverse effects caused by Dex treatment (Luppen et al., 2003).

Aside from defective Wnt/β-catenin signaling, previous studies have shown that Dex can inhibit the expression of IGFs which are major mediators of GH and typical regulators of bone growth. In addition, Dex can alter the synthesis of IGFBPs, which are binding proteins of IGFs (Giustina et al., 2008). IGF-I and -II are known to have the capacity to stimulate osteoblast differentiation (Delany et al., 1994). IGFBP-6 selectively blocks the effect of IGF-II on osteoblasts, whereas IGFBP-3, -4, and -5 stimulate bone cell growth (Conover, 2008). Following Dex treatment, the expression levels of IGFBP-6 has been shown to be increased and the expression levels of IGFBP-3, -4, and -5 are decreased (Bouvard et al., 2010), further illustrating the negative effects of Dex on bone formation.

Moreover, Dex has also been shown to suppress the synthesis of types I and III collagens, leading to a subsequent decrease in the bone matrix available for mineralization (Canalis et al., 2007). Excess use of Dex causes down-regulated expression of some non-collagenous proteins such as OCN and osteopontin (OPN) (Canalis et al., 2007), as well as up-regulated expression of inhibitors of mineralization (e.g., DMP-1 and Phex) (Yao et al., 2008).

Dex-induced skeletal cell apoptosis has been shown to be associated with the activation of caspases 3, 7, and 8 (Espina et al., 2008; O'Brien et al., 2004). These caspases are key mediators of multiple apoptotic signaling pathways such as the Fas/FasL death receptor pathway and Bax/Bcl-2 pathway (Espina et al., 2008). Furthermore, Dex has been shown to increase the expression of Bax (an apoptosis promoter) causing growth plate chondrocyte apoptosis and ablation of Bax was shown to protect mice from Dex-induced growth impairment (Zaman et al., 2012).

Aside from the inhibition of osteoblast differentiation and function, it is evident that Dex has pro-apoptotic effects on osteoblasts and osteocytes. The enhanced apoptosis of osteoblasts has a negative effect on bone formation, whereas the enhanced apoptosis of osteocytes has a strong negative effect on bone strength, without a decrease in bone mass. This is a result of the main function of osteocytes being the sensing of local microdamage and inducing bone repair (Cherian et al., 2008). Dex-induced apoptosis of osteocytes can potentially disrupt the lacunar-canalicular network and lead to the defeat of signal detecting, resulting in an inhibition of damaged bone replacement or local remodeling (Jilka et al., 2013).

Dex-induced effects on osteoclastogenesis

Although the prolonged use of high doses of Dex has been illustrated to enhance osteoclast formation leading to altered bone resorption (Olney, 2009), the molecular mechanisms responsible are yet to be clearly identified. While Dex has been demonstrated to have the capacity to inhibit the expression of some pro-osteoclastic cytokines (e.g. IL-1 and IL-6) in osteoblasts and stromal cells (Hofbauer et al., 2000), it has also been linked to upregulation of

RANKL and M-CSF (Hofbauer and Rauner, 2009) as well as inhibiting OPG (Sasaki et al., 2002). This increase in the RANKL/OPG ratio may directly result in the stimulated osteoclast precursor recruitment, which will translate into increased osteoclast differentiation and a prolonged osteoclast lifespan (Jia et al., 2006). However, the mechanism by which Dex induces osteoclast recruitment and differentiation still remains unclear.

Dex-induced bone marrow adipogenesis

Similar to MTX, Dex has also been shown to modulate expression of a range of osteogenic and/or adipogenic transcription factor to favor adipocyte differentiation at the expense of osteoblast commitment during MSC differentiation (Fig.1-18) (Canalis et al., 2007), which contributes to reduced bone volume with increased bone marrow adiposity (Pereira et al., 2002). It is evident that the transactivation of a crucial adipogenic transcription factor, C/EBPα, could be induced by excess Dex, which in turn increases the expression of PPARγ2 and the promotion of adipogenesis. Meanwhile, Dex also has the capacity to suppress the expression of the essential osteogenic transcription factor Runx2 (Shi et al., 2003). Thus, the adipocyte-osteoblast imbalance caused by Dex may result in concurrent bone marrow adiposity and bone loss.

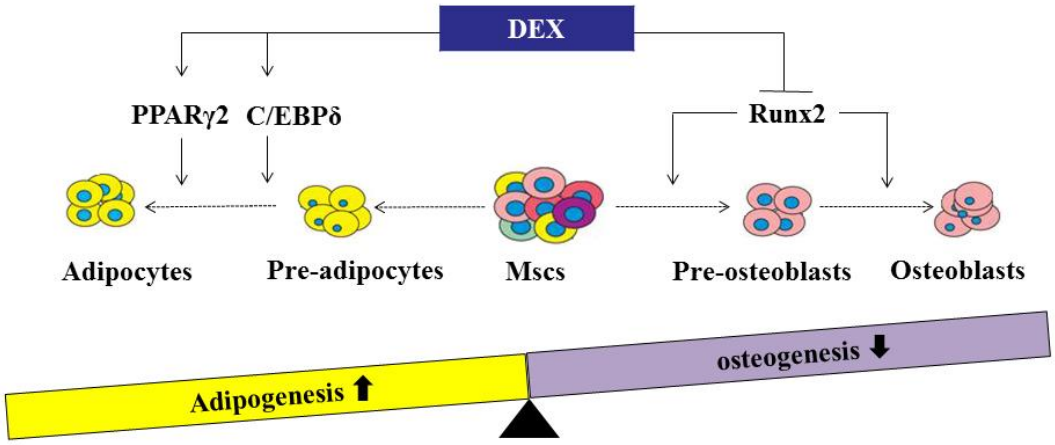


Figure 1-18. Dex-induced bone loss and bone marrow adiposity. Dex could stimulate the expression of adipogenic transcription factors (e.g. C/EBPα and PPARγ2) and inhibit expression of osteogenic transcription factors (e.g. Runx2), resulting increased marrow adiposity and reduced bone volume. Lines with arrowheads indicate stimulatory pathways, and lines with bars indicate inhibitory pathways. Figure was adopted from (Wagner et al., 2010).

1.4.3 Potential Preventive Strategies and Treatment Approaches for Chemotherapy-induced Bone Growth Defects in Childhood

While increasing success has been achieved in paediatric malignancy by intensive use of chemotherapeutic treatment, high prevalence of chemotherapy induced severe adverse effects on the skeleton such as bone growth arrest, low bone mass and associated fractures has been observed in the patients and survivors. Aside from the direct effects of chemotherapeutic agent(s), a number of disease or chemotherapy-related factors, such as nutrition status, life style and even the original disease itself, have been shown to contribute to the observed bone growth defects during or after chemotherapy. However, while the underlying mechanisms for chemotherapy-induced bone growth defects still remain to be understood further, due to the current lack of specific treatments, there is a strong need to develop adjunct preventative treatments for minimizing adverse skeletal effects during/after childhood chemotherapy.

Due to the lack of corresponding early-onset symptoms, chemotherapy induced bone loss is occasionally concealed until the occurrence of fragility fractures. Therefore, the routine monitoring and assessment on the basis of age, sex, body mass index (BMI), BMD and other clinical risk factors are critical for the paediatric patients to maintain skeletal health during/after chemotherapy (Michaud and Goodin, 2006).

A gradual decline in physical activity in cancer patients and/or survivors has been observed owning to individual conditions and disease progression (Ungar et al., 2016). However, the reduction in mechanical loading has been illustrated to be associated with suppressed osteoblast-mediated bone formation and enhanced osteoclast-mediated bone resorption (Takata and Yasui, 2001). Previous studies have shown a tight coupling between physical activity and longitudinal growth in childhood, which is related to the increased mechanical loading (Janz et al., 2010). A regular low-intensity and appropriate physical exercise program is therefore recommended for supporting bone health during and after childhood chemotherapy.

Calcium is evident to be crucial for bone growth and development, particularly in childhood (Peacock, 2010). Long term or intensive use of some chemotherapeutic agents (e.g. Dex and MTX) has been shown to induce aggravated bone resorption which transfers calcium from bone to blood, resulting in the decreased intestinal and renal calcium absorption as well as increased urinary calcium excretion (Michaud and Goodin, 2006). Therefore, increasing calcium intake is suggested to have the potential to reduce bone resorption and maintain bone health (Bonjour, 2011). However, previous studies have demonstrated that calcium

supplementation alone is insufficient to prevent the induced bone loss (Sambrook, 2000). Since vitamin D is well-known to play an essential role in intestinal calcium absorption and bone homeostasis (Peppone et al., 2010; Wei and Giovannucci, 2010), combined supplementation of calcium and vitamin D has been found to have more protective effects on bone metabolism (Homik et al., 2000). Collectively, adequate dietary intake of calcium and vitamin D is recommended to the paediatric patients during/after chemotherapy.

Bisphosphonates are a class of chemical analogs of pyrophosphate which are highly effective against osteoclast-mediated bone loss by inducing osteoclast apoptosis (Rogers et al., 2011). Aside their inhibitory effects on bone resorption, bisphosphonates have been shown to play an important role in stimulating osteoblast proliferation and maturation, leading to the promoted bone formation (von Knoch et al., 2005). In addition, they have been recently prescribed in children with osteogenesis imperfect, juvenile osteoporosis and osteonecrosis (Bianchi, 2005; Kotecha et al., 2010; Ward et al., 2011). Although the absorption of bisphosphonates could be repressed by calcium supplementation, bisphosphonate administration is recommended to be combined with the supplementation of vitamin D and calcium to minimize the risk of hypocalcaemia (Poole and Compston, 2012). Previous studies have also shown that long-term bisphosphonate treatment could be well tolerated by children (Ward et al., 2007), however, toxicity of bisphosphonates (e.g. gastrointestinal toxicity with oral bisphosphonates whilst renal toxicity with IV bisphosphonates) is still a major concern (Guise, 2006). In addition, there have been cautions that intensive and prolonged use of bisphosphonates has the potential to cause an over-suppression of bone turnover and ultimately increase fracture risks (Rauch et al., 2006; Whyte et al., 2008).

MTX is the most commonly used anti-folate antimetabolite in paediatric oncology, and intensive or prolonged usage of MTX treatment is evident to interrupt skeletal regulations and cause bone growth defects (Xian et al., 2007). Folate deficiency has been found in children with ALL receiving MTX treatment (Ulrich and Potter, 2006) and has also been demonstrated to increase chemotherapeutic agent-induced toxicity in rats (Branda et al., 1998). Since folinic acid (also known as leucovorin), a 5-formyl derivative of tetrahydrofolic acid, could be readily converted to active forms of folate in human body to maintain to DNA and RNA synthesis, folinic acid supplementation has been adopted as a part of MTX-related chemotherapeutic regimens in reducing MTX toxicity and widely used in clinical practice (Cohen, 2004; Peters et al., 2000). In particular, recent studies have revealed the efficiency of folinic acid in the preservation of bone formation as well as the suppression of resorption and bone marrow adiposity during MTX exposure (Fan et al., 2009; Fan et al., 2012b; Xian et al., 2008). However, folinic acid treatment-induced increased β-catenin activation in the bone has also been observed in a rat MTX treatment model (Georgiou et al., 2015), which has raised a potential concern against the over or long-term use of folinic acid during MTX chemotherapy as over activation of β-catenin activation in bone has been observed to be associated with osteosarcoma (Kansara et al., 2009). In addition, high dose folinic acid administration has also been shown to be associated with reduced MTX treatment efficacy in childhood ALL, and/or potentially enhanced tumor progression (Robien, 2005; Skarby et al., 2006). Thus, further studies are required to examine the underlying mechanism, clinical efficacy and optimal dosage of folinic acid supplementation for preserving bone growth in paediatric oncology patients.

Since Wnt signaling is evident to act as an important regulator of osteogenesis, manipulating

the Wnt pathway for bone protection appears to be a plausible therapeutic approach (Kim et al., 2013). As reviewed above, Wnt signaling pathway is a multiple-step process which could be modulated by various regulators including secreted Wnt antagonists (e.g. sclerostin and Dkk-1) (Canalis et al., 2007). Consequently, inhibition of these negative regulators to promote the Wnt signaling pathway and therefore stimulate bone formation has arisen as an attractive therapeutic target for the prevention and treatment of bone loss. An anti-sclerostin neutralizing antibody has been under extensive investigation and it has been demonstrated that its administration could strikingly increase bone formation in rats (Li et al., 2009). Promising results have been observed in phase I trial of anti-sclerostin antibody (Padhi et al., 2011). Similarly, previous studies have found that the anti-Dkk-1 antibody has the potential to restore trabecular bone mass and density (Glantschnig et al., 2010). Since Wnt signaling has been shown to be disrupted following MTX and/or Dex treatment, the agents which specifically modulates the Wnt signaling pathway could be investigated as one of the most attractive strategies to prevent or treat chemotherapy-induced bone growth defects.

Recently, natural products have attracted a great attention for their therapeutic potentials in reducing bone loss and maintaining bone health (Su et al., 2017). Genistein, one of the well-known flavonoids, is an estrogen receptor-selective binding phytoestrogen which is a naturally occurring isoflavone abundant in soy (Sureda et al., 2017). Previous studies have shown genistein plays crucial roles in stimulating osteogenesis and suppressing adipogenesis and osteoclastogenesis in ovariectomized rats and humans (Ming et al., 2013). In particular, the supplementation of genistein in MTX treated-rat was found to be associated with preserved osteogenic differentiation, and prevented marrow adiposity and bone loss (King et

al., 2015; Raghu Nadhanan et al., 2013). Although more studies and clinical trials are warranted, genistein could be recommended as a promising natural product for therapies targeting chemotherapy-induced bone defects.

Resveratrol is another naturally-occurring polyphenol present in various plants (e.g. red grapes, peanuts, and so on) (Dai et al., 2007). Due to its anti-oxidant, anti-inflammatory, and anti-catabolic properties, resveratrol has the potential to act as a potent chemo-preventive agent (Cecchinato et al., 2007; Mukherjee et al., 2010). In particular, it has been shown that resveratrol supplementation at 1mg/kg could prevent the enhanced osteoclast and adipocyte formation induced by MTX treatment, and therefore to attenuate MTX-induced defects in the growth plate, primary spongiosa, bone volume, and marrow adipose tissue content (Lee et al., 2017). On the other hand, when resveratrol was administered at a higher dose (e.g. at 10 mg/kg/day), MTX-induced damaging effect on skeleton was found to be markedly aggravated (Lee et al., 2017), suggesting the dose-dependent benefits of resveratrol supplementation in limiting chemotherapy-associated bone defects.

Fish-derived omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are dietary fats with various health benefits (Su et al., 2008). Particularly, previous studies have shown that EPA and DHA could significantly enhance osteoblast formation and activity and repress pro-inflammatory mediators (James et al., 2000; Sun et al., 2003), leading to the stimulated bone development and inhibited inflammatory bone loss (Fong et al., 2012). Since chemotherapy-induced bone growth defects are associated with the upregulation of proinflammatory cytokines in bone, anti-inflammatory fatty acid supplementation has the

potential to counter the inflammatory condition and attenuate chemotherapy-related bone loss (Raghu Nadhanan et al., 2014). However, further studies are required to elucidate mechanisms of specific fatty acids and determine their clinical efficiencies in the chemotherapy setting.

In conclusion, chemotherapy induced growth defects are associated with growth plate dysfunction, aggravated bone resorption, inhibited bone formation and enhanced marrow adipogenesis. However, the current pharmacological anabolic or antiresorptive therapeutic approaches could not cover all the aspects of pathogenesis and the clinical effects are relatively limited. Hence, apart from appropriate monitoring, lifestyle changes, adequate nutritional intake and routine physical exercise, there is an urgent requirement for novel, safe, specific and cost-effective therapeutic strategies preserving bone growth during and after chemotherapy.

1.5 Project Hypothesis and Aims

The scope of the current PhD project is to investigate the potential of specific chemokine(s) to act as molecular linker between the observed induction of skeletal cell apoptosis and stimulated osteoclastic cell formation and activity following MTX and/or Dex therapy, which could enhance our understanding of chemotherapy-associated bone growth defect.

1.5.1 Research Hypothesis

In the current PhD project, it is hypothesized that chondrocyte/osteocyte apoptosis induced by

Dex and/or MTX treatment could trigger the induction of a particular subset of cytokine(s)/chemokine(s), and that these cytokine(s)/chemokine(s) have the potential to promote chondroclast/osteoclast recruitment and formation, resulting in subsequent bone growth defects.

1.5.2 Research Aims

1. Using MTX and/or Dex treatment models in cultured ATDC5 chondrocytes to determine whether specific cytokine(s)/chemokine(s) derived from MTX ± Dex-treatment-induced apoptotic chondrocytes have the capacity to promote osteoclast recruitment and differentiation *in vitro*.

2. Using MTX and/or Dex treatment models in cultured IDG-SW3 mature osteocytes to identify whether the induced osteocyte apoptosis could produce pro-osteoclastic cytokine(s)/chemokine(s) that promote osteoclast migration and formation *in vitro*.

3. Using a model of Dex treatment in young rats with functional blockade for selected cytokine(s)/chemokine(s), which are exhibited to have the most prominent induction following MTX and/or Dex treatment in the above *in vitro* studies, to examine its potential effect on Dex-induced structural, cellular and molecular damage/changes within the bone and marrow microenvironment, and its potential bone protective effect during Dex treatment *in vivo*.

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CHAPTER TWO

STUDY 1: Chemokine CXCL12 as a potential molecular linker between chondrocyte apoptosis and osteoclast recruitment and formation following methotrexate and/or dexamethasone treatment in vitro

Statement of Authorship

Chemokine CXCL12 as a potential molecular linker between chondrocyte apoptosis and osteoclast recruitment and formation following methotrexate and/or dexamethasone treatment *in vitro*

Qian Tang (Candidate)
Planned and carried out all the relevant experiments in the current project. Performed concept, prepared figures, drafted, edited and revised the manuscript. The manuscript is prepared for submission to *J Cell Physiol*.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: …21/09/17……

Cory J. Xian
Contributed to conception of the project, supervision of project development, data interpretation, and critical manuscript evaluation and acted as the corresponding author.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: …21/09/17……

Chemokine CXCL12 as a potential molecular linker between chondrocyte apoptosis and osteoclast recruitment and formation following methotrexate and/or dexamethasone treatment *in vitro*

Qian Tang, Cory J. Xian

Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia;

* Corresponding author: Cory J Xian, PhD

School of Pharmacy and Medical Sciences

University of South Australia

City East Campus, GPO Box 2471

Adelaide 5001, Australia

Tel: +61 8 830 21944

Fax: +61 8 830 22389

Email: Cory.Xian@unisa.edu.au

Abstract

Intensive use of chemotherapeutic agents such as methotrexate (MTX) and dexamethasone (Dex) is known to cause bone growth defects such as growth arrest, bone resorption and bone loss, for which the mechanisms remain unclear. The current study utilized MTX and/or Dex treatment models in cultured ATDC5 chondrocytes to investigate whether MTX ± Dex-treatment-induced apoptotic chondrocytes secrete cytokine(s)/chemokine(s) with the capacity to stimulate osteoclast recruitment and differentiation. After 48 h 10^{-5}M MTX and/or 10^{-6}M Dex treatment in cultured mature ATDC5 chondrocytes, significant cell apoptosis was observed. PCR array analyses of MTX ± Dex-treated chondrocytes identified that chemokine CXCL12 having the most prominent induction in each treatment group, which is confirmed by quantitative RT-PCR analysis in treated cells and ELISA for the conditioned medium from treated cells. In addition, conditioned medium from the treated chondrocytes had the potential to promote migration of RAW264.7 osteoclast precursor cells and formation of osteoclasts, and these promoting effects of the conditioned medium were significantly inhibited by the presence of the neutralizing antibody for CXCL12. These findings suggest that MTX and/or Dex treatment can cause apoptosis of chondrocytes and elevate expression of chemokine(s) particularly CXCL12, which has been identified to promote osteoclast recruitment and formation. Data from current study will be useful for further exploring and understanding the underlying mechanisms of bone defects following MTX and/or Dex therapy.

Key Words

Chemotherapy, Bone defects, CXCL12, Chondrocyte apoptosis, Osteoclasts

Introduction

Although chemotherapeutic agents have been widely used and achieved great success in the management of paediatric malignancies, it has been revealed that intensifying use of chemotherapy could significantly increase the prevalence of long term adverse effects on the skeleton including bone growth defects in childhood, which may persist into adulthood (Canalis et al., 2007; Ecklund et al., 1997; Mandel et al., 2004). For example, while children with acute lymphoblastic leukemia (ALL), the most common childhood malignancy, have an over 80% of survival rate following chemotherapy involving intensive use of methotrexate (MTX) and dexamethasone (Dex) (Mitchell et al., 2009), they have been found to develop a lower bone turnover with lower bone mineral density (BMD) and achieve a considerable decrease in height velocity during and after chemotherapy treatment (Viana and Vilela, 2008). Despite unclear mechanisms, there is evidence to suggest that these defects are associated with altered regulation of the cells residing in the growth plate cartilage (where the hypertrophic chondrocytes are responsible for establishment of calcified cartilage scaffold for future bone formation), bone and marrow microenvironment (where calcified cartilage is resorbed by chondroclasts at the growth plate/metaphysis transitional zone, and bone modelling and bone remodelling in the metaphysis bone are carried out by bone-forming osteoblasts and bone-resorptive osteoclasts) (Xian et al., 2007). Typical cellular disruption such as induction of chondrocyte apoptosis accompanied by stimulated recruitment/differentiation of chondroclasts and/or osteoclasts has been observed after MTX or Dex treatment, which results in uncontrolled bone or cartilage loss, the thinning of the growth plate and the impairment of bone growth (Fan et al., 2012; Fan et al., 2011; Olney, 2009; Zaman et al., 2012). However, the underlying mechanisms particularly the potential intercellular crosstalk(s) among skeletal cells following MTX and/or Dex treatment still remain largely unknown. Due to the lack of clear understanding of the underlying

mechanisms, currently there are no preventive strategies and specific treatments for chemotherapeutic agent-associated adverse skeletal effects.

Despite being poorly understood, a close temporal and spatial coupling between skeletal cell apoptosis and osteoclast recruitment and formation has been illustrated (Cardoso et al., 2009; Kennedy et al., 2012; Shandala et al., 2012). These studies indicate that induced skeletal cell apoptosis may potentially affect the secretion of some regulatory factors that mediate the recruitment and differentiation of osteoclasts. Recently, accumulating evidence has also shown that the skeletal and immune systems interact with each other and have several regulators in common, such as cytokines and chemokines (Caetano-Lopes et al., 2009). A number of cytokines and/or chemokines have been observed to participate in multiple skeletal cell regulation pathways. It has been demonstrated that the cell death of chondrocytes is accompanied with the induction of specific cytokine(s)/chemokine(s) such as tumour necrosis factor-alpha (TNF-α) and chemokine CXCL12 at the chondro-osseous transitional zone (Lotz et al., 1995; Wei et al., 2006) and their increased expression levels may play a vital role in enhanced osteoclastogenesis (Gronthos and Zannettino, 2007; King et al., 2012; Wei et al., 2005; Wright et al., 2005; Yu et al., 2003). Therefore, a specific subset of cytokine(s)/chemokine(s) may be ideal candidates for investigation to elucidate the molecular link between skeletal cell apoptosis and osteoclast differentiation following MTX and/or Dex therapy. Using MTX ± Dex treatment models in cultured mature chondrocytes, the current study examined the treatment effects on induction of chondrocyte apoptosis, expression of cytokines/chemokines and functions of the most induced cytokine(s)/chemokine(s) in osteoclast precursor cell migration and formation.

Materials and Methods

Cell lines and culture conditions

Mouse ATDC5 chondrogenic cells were employed in the current study. Briefly, cells were seeded in basal medium containing alpha minimum essential medium (α-MEM; Sigma, Sydney, NSW, Australia) supplemented with 5% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma), 15 nM Hepes (PH7.4; Sigma), 100 U/ml penicillin/100 μg/ml streptomycin (Invitrogen) in 6-multiwell plates at a density of $5x10^4$ cells/well. They were then be cultivated for two days to reach confluence (day 0) at which point the medium was replaced with chondrogenic differentiation medium, which is basal medium additionally supplemented with 5 ng/ml sodium selenite (Sigma), 10 μg/ml transferrin (Invitrogen) and 300 μM L-ascorbate-2-phosphate (Sigma). Cells were incubated in a humidified atmosphere (37˚C, 5% CO_2) and the medium was changed every second day. In addition, cells were harvested at various time points (days 0, 4, 10 and 20) for identifying stage of chondrocyte hypertrophy and for determining time period for Dex treatment.

Alcian blue staining

To visualize the process of chondrogenic differentiation, ATDC5 cells were stained with Alcian blue as described (Altaf et al., 2006) at various time points (days 0, 4, 10 and 20). In brief, cells were fixed with 100% methanol at -20°C for 20 min, stained with 0.1% Alcian blue 8GX (Waldeck GmbH & Co. KG., Munster, NW, Germany) in 0.1M hydrochloric acid (HCl) overnight. Images were captured and analyzed using an optical microscope. In addition, in order to quantify the intensity of the staining, Alcian blue-stained culture plates were rinsed three times with phosphate-buffered saline (PBS) and extracted with 6M Guanidine-HCl solution overnight at room temperature. Extracted dye was subsequently quantitated by measuring its absorbance at 600 nm by spectrophotometry.

MTX and/or Dex treatment

As a means to assess the effects of MTX ± Dex treatment during hypertrophic chondrogenesis stage, ATDC5 cells were either treated with MTX at 10^{-5}M and/or Dex at 10^{-6}M, which has been shown to cause cell apoptosis as previously described (Kogianni et al., 2004; Shandala et al., 2012), in differentiation medium on day 18 (hypertrophic chondrocyte as determined from the above time course study) for 48 h, or with the normal differentiation medium as a untreated control. Part of -treated and -untreated cells were trypsinised on day 20 for RNA extraction and gene expression analysis.

Generation of conditioned medium

The conditioned medium was firstly collected before MTX ± Dex treatment on day 18. To generate MTX and/or Dex-free conditioned medium after treatment, the -treated cells were rinsed thoroughly with PBS on day 20 and then cultured for additional 24 h with fresh medium, after which the conditioned medium was collected on day 21. In addition, the conditioned medium was also collected from ATDC5 cells without treatment on days 18 and 20, respectively. All conditioned media were spun down at 1500 RCF for 15 min to remove any cell debris and stored at -20 ℃ until further use.

Immunofluorescence analyses

To assess the effects of MTX and/or Dex treatment on apoptotic cell death, immunofluorescence analyses for cleaved caspase-3, the active form of caspase-3 and a key mediator of apoptosis (Budihardjo et al., 1999), were carried out in ATDC5 cells with/without treatment was carried out as described (Heshiki et al., 2015). In brief, following fixation and permeabilization, MTX ± Dex-treated and -untreated cells were successively incubated with primary rabbit anti-cleaved-caspase-3 antibody (1:250; Asp175; Cell

Signaling Technology, Beverly, MA) and then with Alexa Fluor®-conjugated secondary antibody (1:500; Cell Signaling Technology) for 1 h at 37℃, after which the nuclei were stained with 0.5 μg/ml 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Sigma) for 5 min. The density of cleaved caspase-3 positive (cleaved caspase-3^{+}) cells was then calculated as a percentage of DAPI-stained cells by using fluorescence microscope.

Chemokine/Cytokine PCR array

To investigate MTX and/or Dex treatment effects on the chemokine/cytokine expression, quantitative mRNA expression analysis of 84 major cytokines and chemokines was performed using a mouse cytokines & chemokines RT^2 Profiler™ PCR Array Kit (PAMM-150ZD; QIAGEN Science, Germantown, MD) according to the manufacturer's instructions. Briefly, the PCR array was conducted on the Bio-Rad CFX Connect PCR system (Bio-Rad, Hercules, CA) using iTaq Universal SYBR Green Supermix (Bio-Rad). Comparative CT ($2^{-\Delta}$CT) method was used to calculate the levels of relative gene expression to the internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Further conformational verification studies such as real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were performed for the selected prominently induced member(s) (e.g. CXCL12) identified via the PCR array.

Real-time qRT-PCR

Real-time qRT-PCR was performed to verify any expression changes in cytokine(s)/chemokine(s) (e.g. CXCL12) identified by the above PCR array studies. Meanwhile, qRT-PCR was also conducted for molecular analysis of treatment effects on mRNA expression of some major cartilage matrix proteins genes (e.g. type II and type X collagen), apoptosis regulatory molecules (e.g. Bcl-2, Bax, Fas and Fas-L), and osteoclast

differentiation gene such as osteoclastassociated receptor (OSCAR). RNA samples were obtained from collected ATDC5 cells as described above. Total RNA was extracted using RNAqueous®-Micro Kit (Ambion; Applied Biosystems, Mulgrave, VIC). For use as template for PCR amplification, RNA samples were reverse-transcribed using iScript™ Reverse Transcription Supermix for RT-Qpcr kit (Bio-Rad). For quantitative RT-PCR of genes of interest, primers were designed using NCBI Primer-Blast software and obtained commercially (GeneWorks, Thebarton, SA, Australia) (Table.2-1). qRT-PCR assays were conducted in a Bio-Rad CFX connect PCR system and data was analysed using the $2^{-\Delta}$CT method relative to the internal control gene Cyclophilin-A (CycA).

Table 2-1. PCR primer sequences used for relative RT-PCR analysis.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Col-2	GGGCTCCCAGAACATCAGCTACCA	TCGGCCCTCATCTCCACATGATTG
Col-X	GGCAGCAGCACTATGACCCAA	ACAGGCCTACCCAAACGTGAGTCC
Bcl-2	CAGCATGCGACCTCTGTTTG	TCTGCTGACCTCACTTGTGG
Bax	CGAGAGGTCTTCTTCCGTGT	GAGCACCAGTTTGCTAGCAA
Fas	AAGATCGATGAGATCGAGCACA	AAGCTTGACACGCACCAGTCT
Fas-L	GAGCTGTGGCTACCGGTGATAT	ACTCACGGAGTTCTGCCAGTTC
CXCL12	ATCAGTGACGGTAAGCCAGTCA	TGCACACTTGTCTGTTGTTGCT
OSCAR	TGGTGTTGCTTCATTTCCTCAG	GGAAAAGGTTGACCCTGCCT
CycA	CGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG

ELISA

In order to determine whether the observed alterations of mRNA expression persist as protein secretion changes for prominently induced member (e.g. CXCL12) as identified by PCR array, soluble protein concentrations of the cytokine(s)/chemokine(s) in conditioned medium were assessed. In brief, sandwich ELISAs were performed on conditioned medium from

MTX ± Dex-treated and -untreated ATDC5 cells using commercially available kits (R&D Systems, Minneapolis, MN) as previously described (Georgiou et al., 2012).

Osteoclast precursor cell migration assay

To examine the potential of selected cytokine(s)/chemokine(s) in the collected conditioned medium (with the prominent induction following MTX and/or Dex treatment) in attracting osteoclast precursor cell recruitment, mouse RAW 264.7 osteoclast precursor cells were subjected to an *in vitro* chemotaxis assay as described (Al-Dujaili et al., 2011). Briefly, the migration assay was carried out using Boyden chambers with 8.0mm pore size PET membranes (3422; Corning Costar Corp., Corning, NY). RAW264.7 cells were seeded into the upper chambers at a density of $5x10^4$ cells/well and the bottom chambers were filled with conditioned medium from ATDC5 cells with/without MTX ± Dex treatment. Meanwhile, basil medium in the presence or absence of specific recombinant chemokine protein such as recombinant mouse CXCL12 protein (10 nM; MAB350; R&D Systems) was used in control groups as described (Yu et al., 2003). After 6 h incubation at 37 ℃ in 5% CO_2, non-migrated cells on the upper side of the membranes were removed with a cotton swab, whereas migrated cells on lower side were fixed with 10% formalin for 15 min and then stained with 0.5% toluidine blue (Sigma). The densities of migrated cells were quantified by using an optical microscope. In addition, the above migration assay in combination with specific chemokine functional blockage was conducted to further determine whether the migration of osteoclast precursor cells could be inhibited by neutralizing the activities of the specific cytokine(s)/chemokine(s). In these blocking experiments, the selected neutralizing antibodies such as anti-CXCL12 antibody (10 μg/ml; MAB310; R&D Systems) were incubated with the conditioned medium at 37°C for 2 h prior to being added in the bottom chambers. Osteoclast precursor migration assay was subsequently carried out as described above. All assays were

performed in triplicate and repeated 3 times.

Osteoclast formation assay

To investigate whether the specific cytokine(s)/chemokine(s) secreted by MTX ± Dex-treated chondrocytes were pro-osteoclastogenic inducers, RAW264.7 cells were also subjected to an *in vitro* osteoclast formation assay. Since receptor activator of nuclear factor-κB ligand (RANKL) is mandatory for osteoclastogenesis, a baseline stimulus of RANKL was provided to trigger osteoclast formation. RAW264.7 cells were seeded at a density of $5x10^3$ cells/well in triplicate in 96-multiwell plates and cultured with 30 ng/ml RANKL (Peprotech, Rocky Hill, NJ) in basal medium for 2 days. On day 3, different conditioned media including those from MTX ± Dex-treated and -untreated ATDC5 cells were added to the wells in a 1:1 ratio with basal medium, after which the concentration of RANKL was adjusted to maintain at 30 ng/ml. The negative control group received basal medium alone, while the addition of 30 ng/ml RANKL (Peprotech) served as a positive control. Additionally, these two groups in the presence of specific recombinant chemokine protein such as recombinant mouse CXCL12 protein (10 nM; MAB350; R&D Systems) were also employed as additional control groups. The medium was changed on day 7 and histochemical staining of tartrate-resistant acid phosphatase (TRAP; a marker of osteoclasts) was performed on day 9 by using a staining kit (Sigma) to identify osteoclasts or osteoclast-like cells in culture as described (King et al., 2012). TRAP-positive ($TRAP^+$) cells were stained ruby red in colour and $TRAP^+$ cells with three or more nuclei were counted as osteoclasts. The osteoclast formation results were expressed as the density of multinucleated osteoclasts in every well (number/mm^2) using image analysis software (cellSens, Build14116; Olympus Corporation, Shinjuku, Tokyo, Japan). To further investigate the potential involvement of specific cytokine(s)/chemokine(s) in osteoclast formation, similar to the migration assay, the selected neutralizing antibodies

such as anti-CXCL12 antibody (10 μg/ml; MAB310; R&D Systems) were utilized in blocking experiments. These antibodies were incubated with the conditioned medium for 2 h and osteoclast formation assay was subsequently performed in a similar fashion. Apart from TRAP staining, part of cells were harvested and qRT-PCR analysis for OSCAR, a costimulatory regulator of osteoclast differentiation, was performed to evaluate the osteoclastogenesis effects among RAW264.7 cells incubated with conditioned media from MTX ± Dex treated/untreated ATDC5 chondrocytes.

Statistical Analyses

One-way Analysis of Variance (ANOVA) and Tukey's post-hoc were used for time-course chondrogenesis studies, and to investigate the significant differences among MTX ± Dex-treated and -untreated groups using Prims 6 software (GraphPad, San Diego, CA), where a P-value < 0.05 is accepted as being statistically significant. In the figures, the symbols *, ** and *** represented $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Results

Differentiation of ATDC5 chondrocytes in culture

To identify suitable culture time period for hypertrophic (mature) chondrocytes to be treated by Dex, an ATDC5 chondrogenic cell differentiation/maturation time-course study was firstly performed. In order to evaluate chondrogenic differentiation *in vitro*, Alcian blue staining for cartilage matrix proteoglycan and qRT-PCR analyses for typical chondrogenesis markers were conducted. As shown in Fig.2-1A, Alcian blue positive cells (colonies) were not detected until day 4 of culture. The typical nodular structures were observed from day 10, which increased in size and number with time in culture. During the

differentiation/maturation phase between days 10 and 20, Alcian blue-positive cells appeared to expand by the accumulation of cartilage matrix resulting from the ongoing differentiation. In addition, the amount of ex-tractable dye was measured by staining absorbance for the quantity of proteoglycan. There was a progressive and significant increase in Alcian blue absorbance from day 0 to day 20 ($P<0.05$, $P<0.01$ and $P<0.001$ on days 4, 10 and 20, respectively compared to day 0) (Fig.2-1B). Chondrogenic differentiation of ATDC5 cells was further characterized by quantitative mRNA expression analyses of chondrogenesis selective markers (type II and type X collagen). mRNA expression levels of type II collagen were found to be elevated on day 4 (peak level, $P<0.01$ compared to day 0) and day10 ($P<0.05$) and then declined by day 20 (Fig.2-1C). Meanwhile, expression of type X collagen, a marker of chondrocyte hypertrophy, was gradually increased and reached a peak and towards a plateau on day 20 ($p<0.001$ compared to day 0) (Fig.2-1D), suggesting reaching mature hypertrophic stage of the differentiation by day 20.

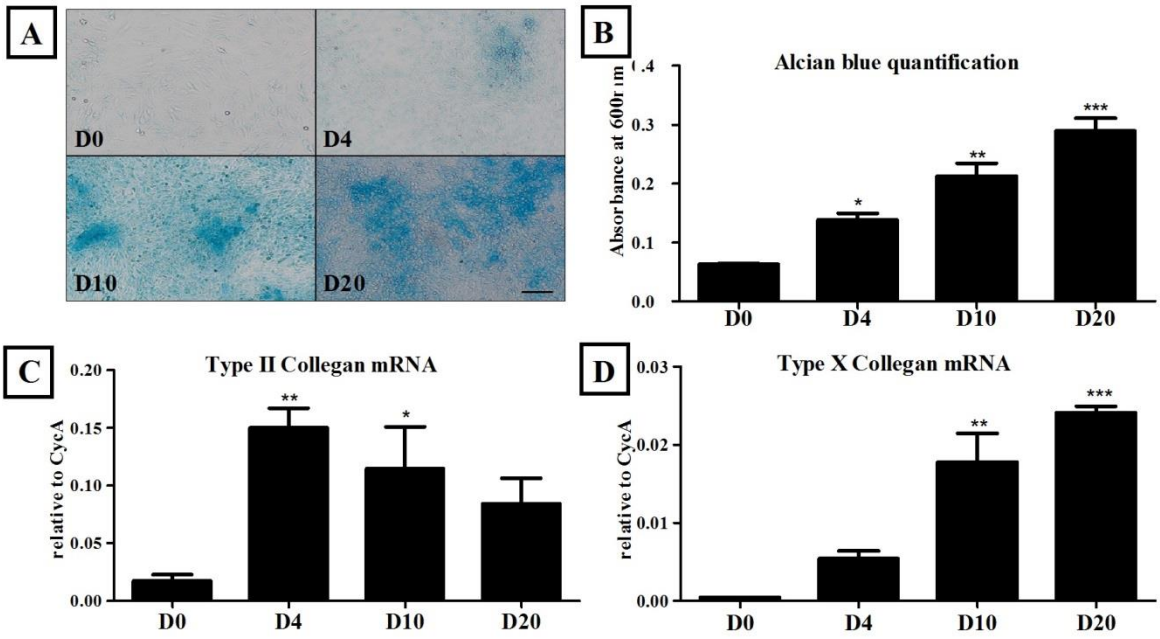


Figure 2-1. Observed changes during ATDC5 chondrogenic differentiation in culture. **(A)** Representative images of Alcian blue staining for ATDC5 cells during time-course (D0-D20) study (days 0, 4, 10 and 20). (**B)** Absorbance values of Alcian blue-stained cells. Quantitative RT-PCR relative gene analyses for chondrogenesis selective markers relative to cyclophilin-A (CycA): **(C)** type II collagen and **(D)** type X collagen. Scale bar on panel A = 400 μm.

Induced chondrocyte apoptosis following MTX and/or Dex treatment

With the above results demonstrating ATDC5 cells reaching hypertrophic stage towards day 20, treatment of ATDC5 cells with/without 48 h MTX (10^{-5} M) and/or Dex (10^{-6} M) was carried out on days 19 and 20 of culture. As a means of assessing the effects of treatment on chondrocyte apoptosis, immunofluorescent analysis for cleaved capase-3, a brief marker for cell apoptosis (Budihardjo et al., 1999), was performed (Fig.2-2A). Quantification of cleaved caspase-3^{+} cells showed a significant increase in apoptosis among MTX ± Dex-treated chondrocytes in comparison to -untreated cells at the same time point (day 20) ($P<0.05$) (Fig.2-2B). Furthermore, qRT-PCR analysis was conducted to investigate the alterations in expression levels of apoptosis regulatory genes which are related to the intrinsic pathway (Bcl-2 and Bax) and the extrinsic pathway (Fas and Fas-L). While Dex treatment appeared to have statistically significant effects on mRNA expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 ($P<0.05$) (Fig.2-2C, D), the Bax/Bcl-2 ratio by which determined the susceptibility of a cell to apoptosis (Salakou et al., 2007) was notably enhanced after Dex alone and Dex + MTX combination treatment ($P<0.05$) (Fig.2-2E). Meanwhile, no significant differences in expressions of Bax and Bcl-2 were found after MTX treatment (Fig.2-2C, D). In addition, although the expression of death receptor ligand Fas-L was maintained in low but detectable levels, a pronounced trend of up-regulation was observed following MTX alone and MTX + Dex combination treatment ($P<0.05$) (Fig.2-2F). However, no significant difference in Fas-L expression was observed in response to Dex treatment. Meanwhile, the expression levels of Fas were found low and showed no obvious changes after MTX ± Dex treatment in the current study (data not shown).

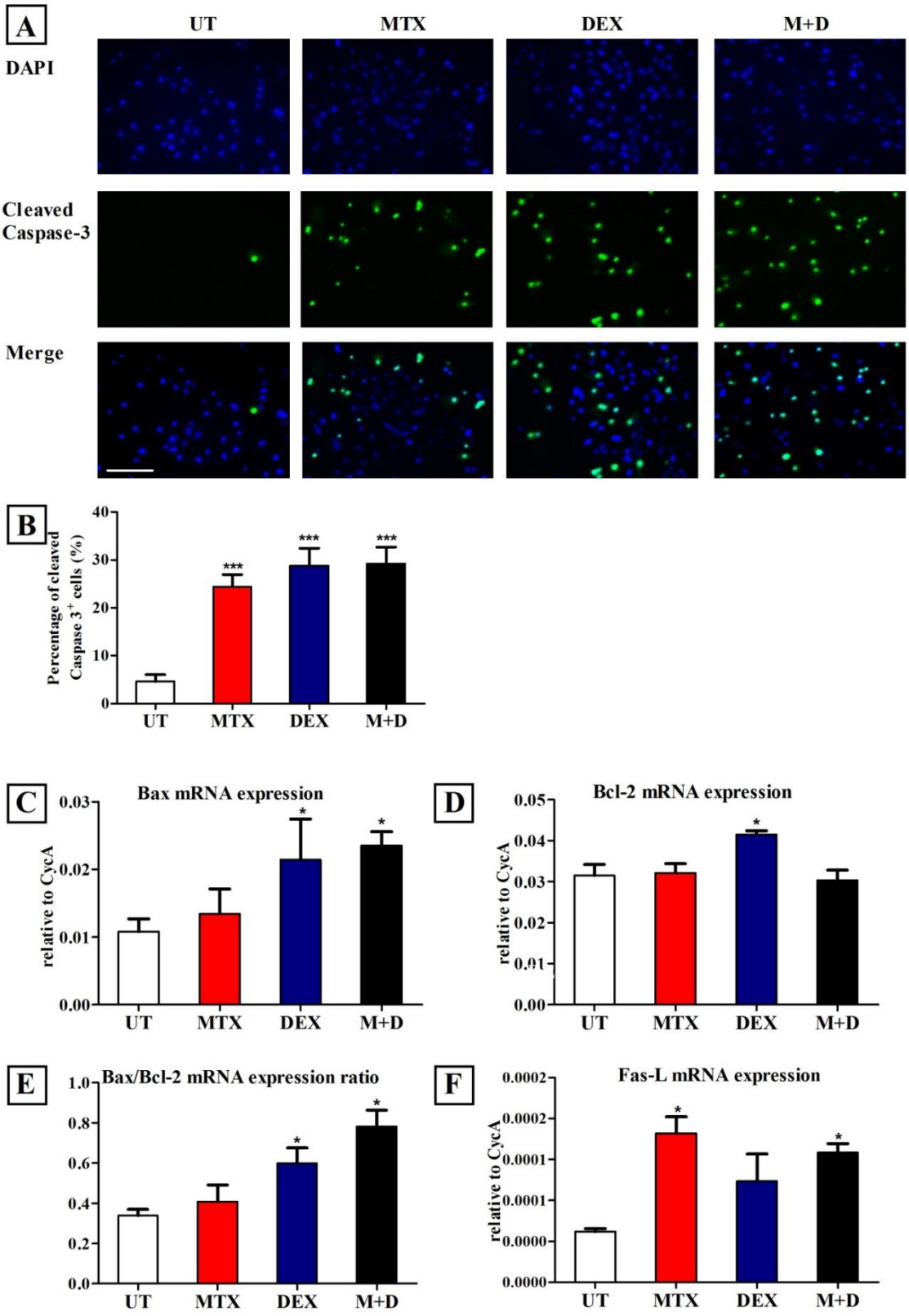


Figure 2-2. Effects of MTX ± Dex treatment on ATDC5 cell apoptosis *in vitro*. (A) Representative images of cleaved caspase-3 immunofluorescent labeling and DAPI labelling for ATDC5 cells in 10^{-5}M MTX, 10^{-6}M Dex, MTX + Dex combination (M+D)-treated, and -untreated (UT) groups. Cleaved Capase-3^{+} positive staining and nuclear marker DAPI are labeled with green and blue, respectively. **(B)** Density measurements of cleaved Capase-3 positive cells. The effects of Dex treatment on mRNA expression of apoptosis regulatory

genes (relative to Cyclophilin-A or CycA): **(C)** Bax, (**D)** Bcl-2, **(E)** Bax/Bcl-2 ratio, and **(F)** Fas-L. Scale bar on panel A = 200 μm.

Prominent induction of CXCL12 in ATDC5 chondrocytes following MTX and/or Dex treatment

To investigate the effects of MTX and/or Dex treatment on expression of the cytokine and chemokine family, a specific PCR array containing 84 major known cytokines and chemokines was conducted. While a number of cytokines and chemokines were found to have upregulated expression levels in response to MTX ± Dex treatment (Fig.2-3A), chemokine CXCL12, which was identified to display the most prominent induction in each treatment group, was selected to be examined further in the current study. In brief, real-time qRT-PCR and ELISA were performed to confirm the observed enhanced CXCL12 expression levels. Using the day 20 samples with/without MTX ± Dex treatment form above ATDC5 time course culture, we found a notable upregulation in CXCR12 mRNA expression ($P<0.01$) following MTX and/or Dex treatment (Fig.2-3B). Consistent with the induced mRNA expression levels, the protein concentrations of CXCL12 in the conditioned medium within all treatment groups were also significantly increased when compared with those of the conditioned medium in untreated group ($P<0.001$) (Fig.2-3C).

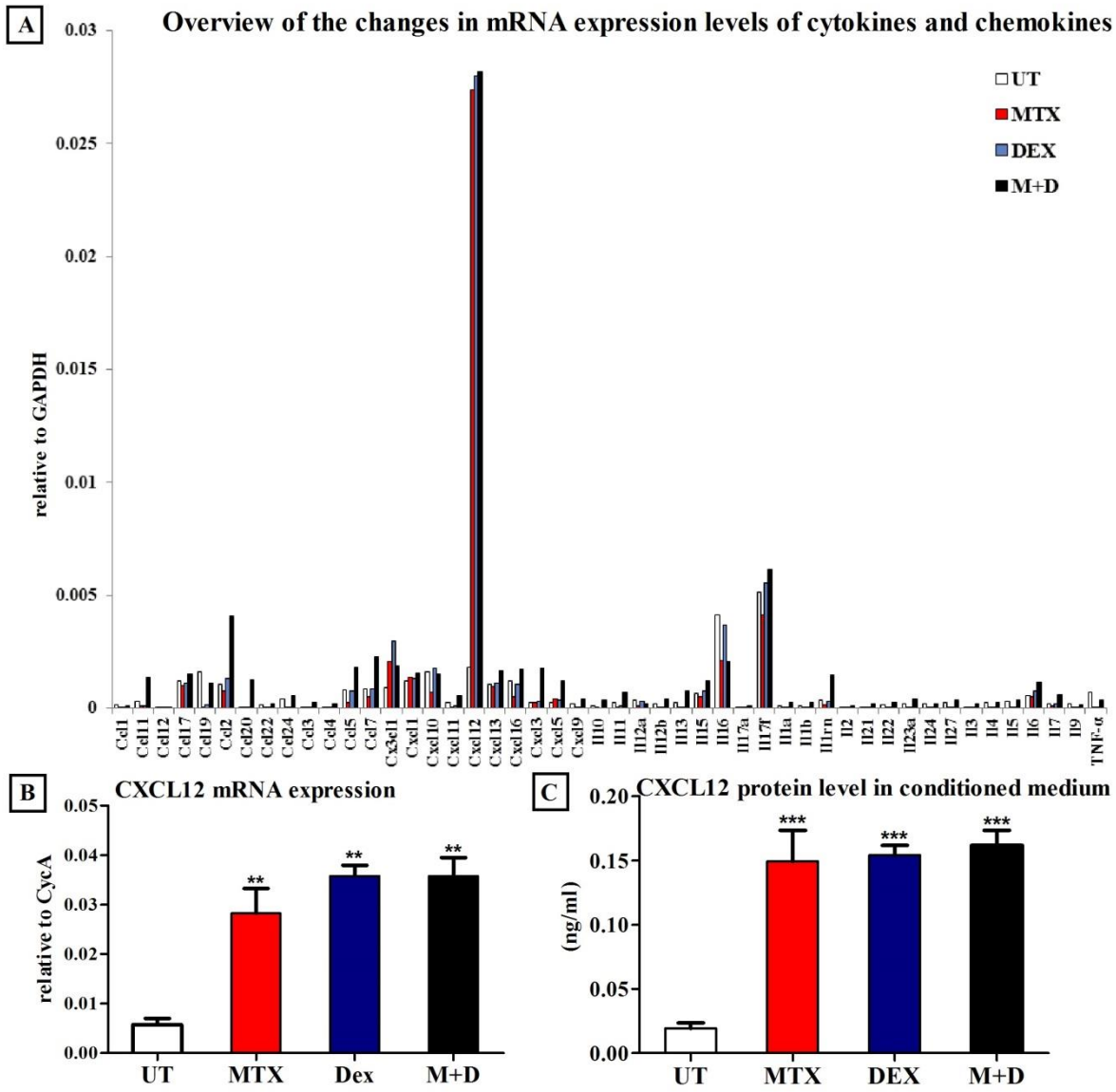


Figure 2-3. Effects of MTX and/or Dex treatment on CXCL12 expression in ATDC5 chondrocytes. (A) Overview of mRNA expression levels of cytokine and chemokine family. CXCL12 was identified to achieve the most prominent induction following 10^{-5}M MTX, 10^{-6}M Dex, and MTX + Dex combination (M+D) treatment when compared to the untreated (UT) control. **(B)** Quantitative RT-PCR relative gene expression analysis for CXCL12 among MTX ± Dex treated/untreated ATDC5 chondrocytes. **(C)** CXCL12 protein concentrations (ng⁄ml) as determined by ELISA in MTX ± Dex-treated/untreated cell conditioned medium.

The potential of CXCL12 in conditioned medium from MTX and/or Dex-treated ATDC5 chondrogenic cells in promoting osteoclast precursor cell migration

To examine the potential effects of MTX and/or Dex-induced induction of CXCL12 on osteoclast recruitment, osteoclast precursor RAW264.7 cells were subjected to an *in vitro*

migration assay applied with different media including those containing conditioned medium from ATDC5 chondrogenic cells with/without MTX ± Dex treatment (Fig.2-4A). Osteoclast precursor cells exhibited significantly augmented migration in the presence of exogenous recombinant CXCL12 protein in basal medium ($P<0.05$) (Fig.2-4A). It was also observed that the conditioned medium from MTX ± Dex-treated-ATDC5 cells has the capacity to stimulate cell migration (($P<0.05$, $P<0.01$ and $P<0.01$ in MTX, Dex, MTX + Dex treatment groups, respectively compared to untreated control) (Fig.2-4A, B). In addition, this promoting response could considerably inhibited by the presence of a neutralizing anti-CXCL12 antibody in each treatment group ($P<0.05$) (Fig. 2-4B).

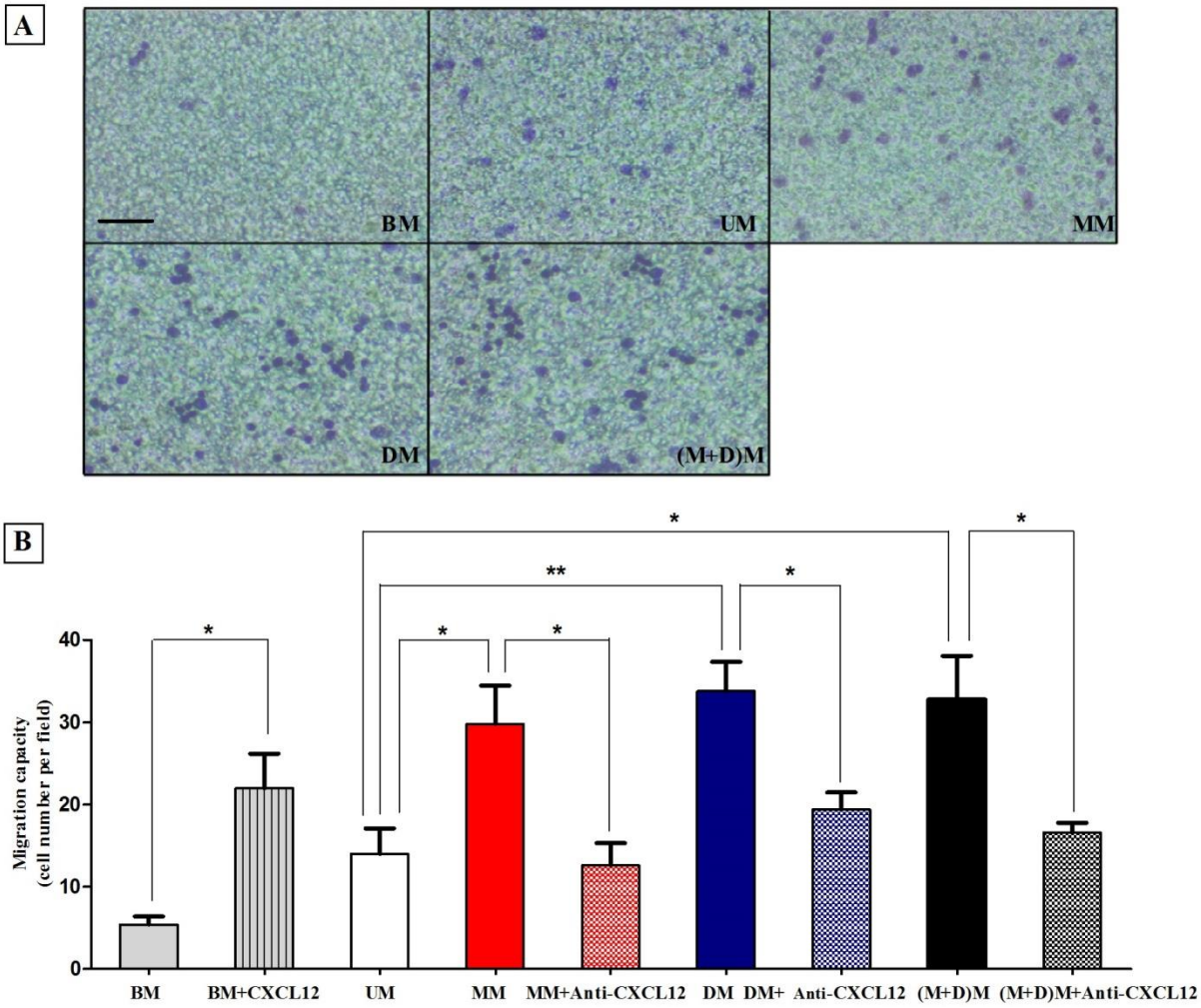


Figure 2-4. Potential of CXCL12 contained in MTX ± Dex-treated ATDC5 chondrocyte conditioned medium in promoting osteoclast precursor cell migration. *In vitro* osteoclast migration assay was carried out with RAW264.7 cells incubated with: basal medium alone

(BM); conditioned medium from the -untreated ATDC5 cells (UM); conditioned medium from MTX (10^{-5}M)-treated ATDC5 cells (MM); conditioned medium from Dex (10^{-6}M)-treated ATDC5 cells (DM); and conditioned medium from MTX (10^{-5}M) and Dex (10^{-6}M) combination-treated ATDC5 cells (M+DM). Meanwhile, BM supplemented with 10 nM recombinant CXCL12 protein (BM+CXCL12) was also used as a control groups; and MM, DM, and (M+D)M additionally added with 10 μg/ml neutralizing anti-CXCL12 antibody were used in blocking experiments. Migrated cells were detected by toluidine blue staining **(A)** and the densities of migrated cells were quantified and illustrated in **(B)**. Scale bar on panel A = 200 μm.

The potential of CXCL12 in conditioned medium from MTX and/or Dex-treated ATDC5 cells in promoting osteoclast formation

Apart from the migration assay, osteoclast precursor RAW264.7 cells were subjected to an *in vitro* formation assay applied with different media. As shown in Fig.2-5A, osteoclast differentiation could not be induced without the presence of RANKL in basal medium. In addition, no statistically significant differences were observed between basal medium alone and basal medium supplemented with 10nM recombinant CXCL12 protein in the absence of RANKL, although the CXCL12 supplementation showed a trend of promoted osteoclastogenesis when compared to basal medium alone. The CXCL12 supplementation in combination with RANKL significantly enhanced osteoclastogenesis when compared to CXCL12 alone ($P<0.01$). These findings suggested that exogenous recombinant CXCL12 protein exerts no or weak direct effects on osteoclast formation in current model. Interestingly, conditioned medium from MTX ± Dex-treated chondrocytes could significantly stimulate the observed osteoclast formation when compared to the conditioned medium from the untreated cells (Fig.2-5A), as observed by formation of a greater number of multinuclear cells with TRAP positivity in the culture (Fig.2-5B) and the significantly upregulated mRNA expression levels of OSCAR in RAW264.7 cells incubated with MTX ± Dex treated conditioned medium (Fig.2-5C). Similar to the findings in the migration assay, these promoting effects of the conditioned medium were considerably repressed after CXCL12

blockade by using the neutralizing antibody for CXCL12 (P<0.001, P<0.01 and P<0.001 in MTX, Dex, MTX + Dex treatment groups, respectively when compared to untreated control) (Fig.2-5B).

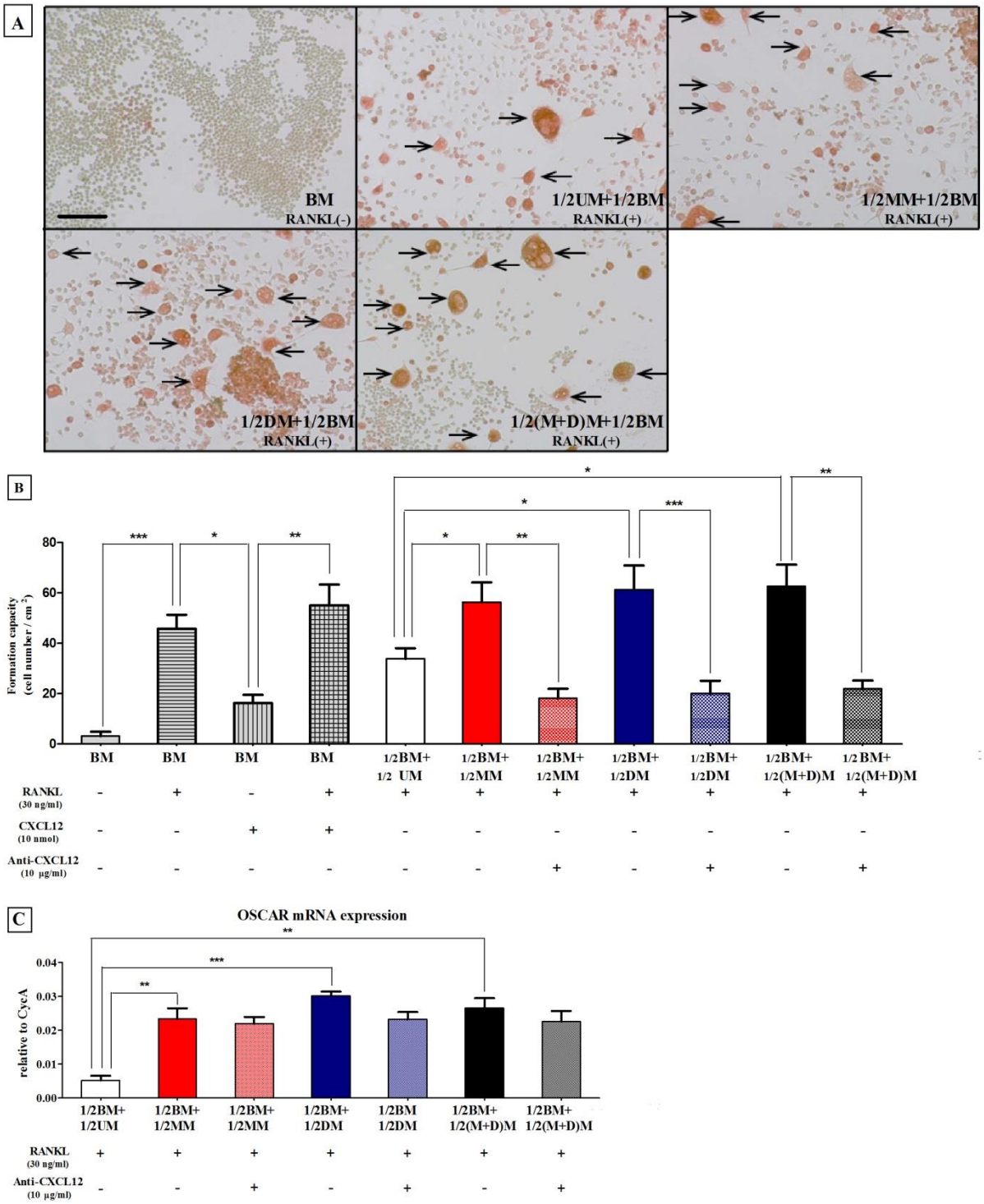


Figure 2-5. Potential of CXCL12 contained in conditioned medium from MTX ± Dex-treated ATDC5 chondrocytes in promoting osteoclast formation. *In vitro* osteoclast formation assay was carried out with RAW264.7 cells incubated with different media: Basal medium (BM) alone; BM supplemented with RANKL at 30ng/ml; BM supplemented with

recombinant CXCL12 protein at 10nM; BM supplemented with RANKL and recombinant CXCL12; Mixture of conditioned medium from the untreated ATDC5 cells (UM) in a 1:1 ratio with BM (1/2UM+1/2BM) supplemented with RANKL; Mixture of conditioned medium from 10^{-5}M MTX-treated cells (MM) in a 1:1 ratio with BM (1/2MM+1/2BM) supplemented with RANKL; (1/2MM+1/2BM) supplemented with RANKL and neutralizing anti-CXCL12 antibody at 10 μg/ml; Mixture of conditioned medium from 10^{-6}M Dex-treated cells (DM) in a 1:1 ratio with BM (1/2DM+1/2BM) supplemented with RANKL; (1/2DM+1/2BM) supplemented with RANKL and neutralizing anti-CXCL12 antibody; Mixture of conditioned medium from 10^{-5}M MTX + 10^{-6}M Dex combination-treated cells ((M+D)M) in a 1:1 ratio with BM (1/2(M+D)M+1/2BM) supplemented with RANKL; and (1/2(M+D)M+1/2BM) supplemented with RANKL and neutralizing anti-CXCL12 antibody. Osteoclasts were detected by TRAP staining and cells with three or more nuclei were depicted by arrows in **(A)**. Osteoclast densities were quantified and illustrated in **(B)**. **(C)** Quantitative RT-PCR relative gene expression analyses for osteoclast-associated receptor (OSCAR) among RAW264.7 cells incubated with conditioned media from MTX ± Dex treated/untreated ATDC5 chondrocytes. Scale bar on panel A = 400 μm.

Discussion

Bone growth during childhood is a multifaceted process which is dependent on the regulation of proliferation, differentiation, activity, and apoptosis of skeletal cells (Xian et al., 2007). Intensive use of MTX or Dex treatment has been shown to considerably interrupt the steady-state regulation of these cells and result in the damage to the skeleton, including bone growth arrest and bone loss in growing long bones (Canalis et al., 2007; Ecklund et al., 1997; Mandel et al., 2004). Although the underlying mechanisms remain unclear, based on previous studies, it is suggested that cytokine(s)/chemokine(s) derived from apoptotic growth plate chondrocytes following MTX and/or Dex treatment have the potential to promote osteoclast/chondroclast recruitment and differentiation at the chondro-osseous junction between the growth plate and metaphysis bone. The above effects could subsequently cause uncontrolled bone or cartilage loss. The current study utilized MTX ± Dex treatment models in cultured ATDC5 chondrocytes to delineate whether MTX ± Dex treatment-induced chondrocyte apoptosis could potentially up-regulate chemokine expression as well as enhance osteoclast migration and formation. Herein we used an ideal *in vitro* chondrogenesis model

and treated mature ATDC5 chondrocytes with 10^{-5} M MTX and/or 10^{-6} M Dex for 48 h. MTX ± Dex treatment was found to induce chondrocyte apoptosis and significantly up-regulate the expression levels of cytokine(s)/chemokine(s) particularly chemokine CXCL12. In addition, conditioned medium from MTX and/or Dex-treated chondrocytes (with a higher content of CXCL12 protein) considerably enhanced osteoclast precursor cell migration and osteoclast formation. These effects could be inhibited by the presence of the neutralizing anti-CXCL12 antibody. These novel findings indicated that apoptotic chondrocytes play a vital role in coordinating osteoclast recruitment and differentiation, potentially via induction of chemokine CXCL12 in the apoptotic cells.

A feasible in vitro model for mature chondrocyte MTX and/or Dex treatment studies

During normal endochondral ossification, hypertrophic chondrocytes in growth plate are observed to mineralize their surrounding cartilage matrix, acting as a scaffold for future bone formation (van der Eerden et al., 2003), and subsequently undergo apoptosis (Green, 1998). As previous studies have confirmed that MTX or Dex treatment could significantly induce chondrocyte apoptosis, which is principally in terminally differentiated hypertrophic chondrocytes (Chrysis et al., 2003; Xian et al., 2007; Zaman et al., 2012), it is necessary to appropriately assess the process of chondrocyte differentiation in culture and find a suitable time point for the following MTX ± Dex treatment studies with cultured mature chondrocytes. ATDC5 cell line was selected in our study as it was illustrated to exhibit the entire spectrum of chondrogenesis and be a practical *in vitro* model for deciphering the molecular mechanism of endochondral ossification (Shukunami et al., 1997). Since Alcian blue is a tetra-cationic dye commonly used for distinctively staining chondrocyte-produced proteoglycan which consists of characteristic polysaccharide chains (glycosaminoglycans) (Rupertus et al., 2011), the process of chondrogenesis could be evaluated by histochemical

analysis of the presence of cartilage glycosaminoglycans by using Alcian blue staining (Terry et al., 2000). In the current study, the observed obvious increase in Alcian blue staining and absorbance reading starting from day 4 upon differentiation culture represented the enhanced presence of the cartilage-specific proteoglycan in the culture. In addition, as the major proteins in cartilage matrices and bones, collagens play an essential role in regulating the size, shape, and strength of skeleton, several types of which are expressed at distinct times and places during chondrocyte differentiation (Gordon and Hahn, 2010). Type II collagen is demonstrated to be the predominant component of cartilage and present in all zones of the growth plate (Zannettino et al., 2005). Despite the diverse expression levels in the various differentiation stages (Rabie et al., 2003), the synthesis of type II collagen has been shown to commence at the early step of chondrogenesis (Zuscik et al., 2008). On the other hand, chondrocyte hypertrophy is confirmed to be accompanied by the reduction of type II collagen and the increased expression of type X collagen, which is examined to be limited in hypertrophic zone (Ballard and Ballard, 1995). Therefore, type II and type X collagen are the ideal molecular markers for assessing chondrogenesis at different stages. We observed significant increased expression levels of type II collagen on days 4 and 10 and declining on day 20, indicating chondrogenic cells may undergo active phrase of differentiation from during days 4 and 10, as well as late phrase toward and/or from day 20. In contrast to the dynamic changes of type II collagen expression levels, it was observed that type X collagen expression gradually increased and peaked and also appeared to plateau on day 20, suggesting late-stage of chondrogenic differentiation (maturation) around day 20. These above findings validated the mature process of ATDC5 cells in culture, from undifferentiated to proliferative, and then finally into hypertrophic chondrocytes. Chondrocyte hypertrophic conversion is defined as the transformed morphological form of chondrocytes from a fibroblastoid to round shape during chondrogenesis (Mackie et al., 2011), which is a unique

and mandatory step allowing subsequent calcification, apoptosis and vascularization (Babarina et al., 2001). For the following *in vitro* parts of the study, MTX ± Dex treatment was conducted during the late stage of chondrogenic differentiation (between days 18-20).

MTX and/or Dex treatment induced chondrocyte apoptosis

Apoptosis is the default fate of most nucleated cells including the growth plate chondrocytes (Green, 1998). Apart from appropriate chondrocyte proliferation and production of calcified cartilage scaffold, bone growth also relies on a balance between survival and apoptosis of chondrocytes within the growth plate (Mackie et al., 2011). Although chondrocyte apoptosis is evident to play a major role in the shaping of long bones during development, the adverse effects of MTX and Dex therapy on the skeleton are induced in part by an increase in chondrocyte apoptosis, which is associated with thinning of the growth plate and bone growth impairment (Xian et al., 2007; Zaman et al., 2012). In addition, two major signaling pathways are well-characterized to be responsible for glucocorticoid-induced chondrocyte apoptosis, including the intrinsic pathway, where the release of apoptogenic protein cytochrome c from mitochondria is regulated by members of the Bcl-2 family proteins (Green and Kroemer, 2004), and the extrinsic pathway, where by the interaction between death receptor Fas and its ligand Fas-L induces apoptosis (Friesen et al., 1999). Previous studies have shown that high-dose corticosterone treatment *in vivo* could stimulate chondrocyte apoptosis via the suppression of Bcl-2 expression and the induction of Bax expression (Mocetti et al., 2001). It also has been demonstrated that Dex-activated pro-apoptotic protein Bax in chondrocytes plays a central role in the pathogenesis by triggering undesired apoptosis and premature loss of growth plate chondrocytes (Zaman et al., 2012). In the current study, immunofluorescent analyses showed that apoptosis of chondrocytes is indeed induced in response to MTX and/or Dex treatment. Although the expression levels of pro-apoptotic protein Bax and anti-

apoptotic protein Bcl-2 were both significantly up-regulated in current Dex treatment model, the relative concentration of Bax and Bcl-2, which serves as a rheostat to determine cell susceptibility to apoptosis (Salakou et al., 2007), was found to increase sharply upon Dex treatment. Meanwhile, no induction in anther apoptosis inducer Fas-L was observed after Dex treatment. These results suggested that Dex-induced chondrocyte apoptosis may particularly through the intrinsic apoptotic pathway in this *in vitro* model. On the other hand, no significant differences in Bax and Bcl-2 expressions were observed after MTX treatment. However, it was shown that MTX treatment caused a notable induction in the expression of Fas-L despite the lack of induction in Fas expression, indicating chondrocyte apoptosis induced by MTX treatment may be regulated by extrinsic pathway, which is in accord with the findings from previous *in vivo* studies (Xian et al., 2007). Nevertheless, further studies are still required to investigate the mechanisms involved in chondrocyte apoptosis after MTX and/or Dex treatment.

CXCL12 displayed the most prominent induction in MTX and/or Dex treatment-induced apoptotic chondrocytes

During normal endochondral ossification, following chondrocyte apoptosis, the calcified cartilage is penetrated by blood vessels from the adjacent primary spongiosa region of the metaphysis (Ballock and O'Keefe, 2003), establishing the channels for invasion of highly specialized cells such as osteoclasts and osteoblasts (Mackie et al., 2011). Osteoclasts identified within growth plate/metaphysis transitional zone are characterized as chondroclasts (Sims and Johnson, 2012) which are specifically involved in the resorption of calcified cartilage (Vu et al., 1998). Consequently, primary trabeculae consisting of the spicules containing both mineralized cartilage and bone matrix are formed in the primary spongiosa, and further remodeled into secondary trabeculae in the secondary spongiosa (Kronenberg,

2003). While intercellular communication within the skeletal microenvironment is well-characterized to be crucial for the above process (Sims and Walsh, 2012), intensive use of MTX and Dex treatment has been shown to cause both direct and indirect interruption of skeletal homeostasis, resulting in severe side effects (Canalis et al., 2007; Xian et al., 2007). It is speculated that there may exist interdependent relationships among some aspects of altered skeletal metabolism following MTX ± Dex treatment such as induced growth plate chondrocyte apoptosis as well as enhanced chondroclast and/or osteoclast recruitment/differentiation through specific intercellular signal cross-talk(s). In recent years, a number of cytokines and chemokines have been demonstrated to act as the essential mediators involved in chemotaxis, differentiation, function and survival of skeletal cells either in physiological or in pathological conditions (Gronthos and Zannettino, 2007; Wei et al., 2010; Wright et al., 2005; Yu et al., 2003). Cytokines are a broad category of messenger proteins for specific biological functions, while chemokines are a large family of small chemotactic cytokines (Almeida et al., 2007), and thus specific cytokine(s)/chemokine(s) could be considered as an ideal candidate serving as a potential intercellular cross-talk molecular link within the skeletal microenvironment. In the current study, it is hypothesised that chondrocyte apoptosis following MTX ± Dex treatment may trigger the induction of specific cytokine(s)/chemokine(s), which could potentially stimulate osteoclast recruitment and differentiation. The expression levels of most of all known cytokines and chemokines in chondrocytes after MTX and/or Dex treatment were systematically examined using a PCR array. Consistent with the findings in previous studies (Andreas et al., 2009; Borzi et al., 2004; Young et al., 2001), a number of cytokines and chemokines were observed to achieve elevated expression after treatment. Among all the examined cytokines and chemokines, chemokine CXCL12 was identified to display the most dominant induction in ATDC5 chondrocytes following MTX and/or Dex treatment, suggesting apoptotic chondrocyte-

derived CXCL12 may be a major player in the current *in vitro* model. The upregulation of CXCL12 was also supported by conformational experiments that both its mRNA expression levels and the protein concentrations in conditioned medium from MTX ± Dex-treated chondrocytes were notably increased. Therefore, CXCL12 was selected as the candidate of interest and was pursued in subsequent studies.

Apoptotic chondrocyte-derived CXCL12 is one of the determinants for osteoclast recruitment and differentiation following Dex treatment

Emerging evidence has shown that CXCL12 plays a crucial role in governing numerous homeostatic, developmental, and pathological processes within the skeleton. It is demonstrated that CXCL12 is capable of eliciting deleterious effects in cartilage under pathological circumstance via upregulation of type X collagen and matrix metalloprotease-13 (MMP-13), leading to stimulated chondrocyte hypertrophy and articular cartilage degradation (Wei et al., 2010). Previous studies have also confirmed that CXCL12 and its unique receptor CXCR4 are expressed in a complementary pattern in the growth plate and the cell death of articular chondrocytes is accompanied by high concentration of CXCL12 at the chondro-osseous transitional zone (Wei et al., 2006). In addition, CXCL12 is observed to play a complementary role in osteoclast precursor cell recruitment and development (Gronthos and Zannettino, 2007). It is illustrated that CXCL12 could significantly stimulate the chemotactic recruitment and migration of circulating osteoclast precursor partly via upregulation of matrix metalloprotease-9 (MMP-9), which is an enzyme obligatory for cells to penetrate the underlying basal lamina barrier in the process of their migration (Yu et al., 2003). Furthermore, it is evident that CXCL12 exert its direct effect on osteoclast differentiation, particularly during the early stage, via elevation of precursor cell number as well as promotion of multinucleated cell fusion, size, and TRAP activity (Wright et al., 2005).

In the current study, in an effort to address the potential role of CXCL12, derived from apoptotic chondrocytes following MTX and/or Dex treatment, in inducing osteoclastogenesis, conditioned medium from MTX ± Dex-treated and/or -untreated chondrocytes was added to cultured osteoclast precursor cells. It was found that both exogenous recombinant CXCL12 protein and the CXCL12 contained in the conditioned medium from MTX, Dex, or their combination-treated chondrocytes (containing 0.149±0.032, 0.154±0.011, and 0.161±0.014 ng/ml CXCL12 protein, respectively) have the capacity to act as a chemoattractant for osteoclast precursor cells *in vitro*. On the other hand, CXCL12 alone (in the absence of RANKL) was found to have no or only a weak capacity to trigger osteoclast differentiation in culture. However, in the presence of RANKL, conditioned medium from MTX, Dex, or their combination -treated chondrocytes considerably promoted osteoclast formation, to the extents of 166.7%, 181.5%, 185.2% higher than untreated cell conditioned medium, respectively. In particular, inhibition of these promoting effects was also observed by the presence of neutralizing antibody for CXCL12 in each treatment group, further confirming the role of CXCL12 in the conditioned medium in enhancing the osteoclastogenic effect in the presence of RANKL.

MTX and Dex are the two most commonly and intensively used agents in childhood oncology, particularly their combination therapy is the key component of the chemotherapy regimen for ALL maintenance treatment in children (Veerman et al., 2009). Although it has been well-established that intensifying MTX/Dex monotherapy could cause bone defects, it is still unclear whether the skeleton impairments induced by MTX combination therapy with Dex are influenced by the synergistic or antagonistic effects of these two agents, leading to the inability to fully understand the underlying mechanisms and provide effective management strategies. Although MTX ± Dex treatments exhibit no differences in inducing

chondrocyte apoptosis and stimulating osteoclastogenesis in current *in vitro* studies, *in vivo* studies should be performed to provide the comparative profiles of MTX and/or Dex treatment in causing the skeletal defects. In addition, further work is still required to reveal the underlying mechanisms (e.g. interaction between apoptotic chondrocyte-derived CXCL12 and receptor CXCR4) for intracellular communication for osteoclast recruitment and formation. Further studies are also required to examine potential involvement of other signaling pathways for the crosstalk between apoptotic chondrocytes and osteoclast/chondroclast recruitment.

Conclusion

In summary, the current study showed that MTX and/or Dex treatment does cause alterations in crosstalk among the skeleton cells, and demonstrated the association between induced chondrocyte apoptosis and enhanced osteoclast migration and formation which could be potentially or at least partially linked molecularly by the induced chemokine CXCL12 in the MTX ± Dex-treated chondrocytes. It is likely that such effects could result in aggravated calcified cartilage or bone resorption, leading to the bone growth defects. Due to the lack of treatments for minimizing the chemotherapy-induced bone growth defects and since CXCL12 derived from apoptotic chondrocytes has the potential to promote osteoclast/chondroclast recruitment and differentiation at the chondro-osseous junction of the growth plate/metaphysis following MTX and/or Dex treatment, CXCL12 may be the ideal candidate for our future investigations to develop therapeutic strategies for preventing or treating bone growth defects in children receiving chemotherapy.

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Disclosure

The authors declared that they have no conflict of interests to this work.

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CHAPTER THREE

STUDY 2: Chemokine CXCL12: a novel osteoclast stimulating factor expressed by apoptotic osteocytes following methotrexate and/or dexamethasone treatment in vitro

Statement of Authorship

Chemokine CXCL12: A novel osteoclast stimulating factor Expressed by apoptotic osteocytes following methotrexate and/or dexamethasone treatment *in vitro*

Qian Tang (Candidate)
Planned and carried out all the relevant experiments in the current project. Performed concept, prepared figures, drafted, edited and revised the manuscript. The manuscript is prepared for submission to *Bone*.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: …21/09/17……

Cory J. Xian
Contributed to conception of the project, supervision of project development, data interpretation, critical manuscript evaluation and acted as the corresponding author.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: …21/09/17……

Chemokine CXCL12: a novel osteoclast stimulating factor expressed by apoptotic osteocytes following methotrexate and/or dexamethasone treatment *in vivo*

Qian Tang, Cory J. Xian

Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia;

* Corresponding author: Cory J Xian, PhD

School of Pharmacy and Medical Sciences

University of South Australia

City East Campus, GPO Box 2471

Adelaide 5001, Australia

Tel: +61 8 830 21944

Fax: +61 8 830 22389

Email: Cory.Xian@unisa.edu.au

Abstract

Methotrexate (MTX) and/or dexamethasone (Dex) are often intensively used in childhood oncology which is known to causes significant bone growth defects. However, although intensive chemotherapy is evident to disrupt steady-state of cell regulation within bone and bone marrow microenvironment, including induced osteocyte apoptosis and increased osteoclast recruitment, leading to significant bone loss, the underlying molecular mechanisms still remain largely unknown and there are no effective bone protective treatments. The current study used *in vitro* MTX and/or Dex treatment models in cultured IDG-SW3 mature osteocytes to address the hypothesis that the induced osteocyte apoptosis could produce pro-osteoclastic cytokine(s)/chemokine(s) that promote osteoclast recruitment and formation. Exposure with MTX at 10^{-5}M, Dex at 10^{-6}M, and MTX + Dex in combination for 48 hours in IDG-SW3 osteocytes (at the mature stage expressing high levels of DMP1 and sclerostin) caused apoptosis which was associated with induction of Bax and Fas-L. The cytokine/chemokine expression PCR array analyses revealed the most prominent induction of chemokine CXCL12 after MTX and/or Dex treatment, which was confirmed by qRT-PCR assay in treated cells and by ELISA of the cell conditioned medium. The conditioned medium from MTX ± Dex-treated osteocytes was found to enhance migration of RAW264.7 osteoclast precursor cells and their osteoclast formation, and these stimulating effects of the conditioned medium were significantly repressed by neutralizing the function of CXCL12. Findings in this study suggested that CXCL12 derived from apoptotic osteocytes following MTX and/or Dex treatment could be an important factor for osteoclast recruitment and bone loss.

Key Words

Chemotherapy, Bone loss, CXCL12, Osteocyte apoptosis, Osteoclasts

Introduction

Although chemotherapy has been widely used for pediatric malignancy treatment, both clinical and experimental studies have revealed that intensive chemotherapeutic treatment could induce severe defects to the bones (Fan et al., 2009; Georgiou et al., 2012a; Mandel et al., 2004; Xian et al., 2007). It has been demonstrated that these defects are associated with the disruptions to steady-state regulation of skeletal cells (e.g. induction of osteocyte apoptosis and enhanced osteoclast differentiation), leading to the reductions of bone mass and bone strength (Fan et al., 2012; Xian et al., 2007). However the underlying mechanism by which chemotherapy induces these bone defects still remains unclear. Consequently, there is a lack of an effective, specific and safer adjunct treatment for the protection of bone during and/or after chemotherapy.

While previous studies have found a tight spatial and temporal relationship between osteocyte apoptosis and osteoclast recruitment/formation following chemotherapy (Shandala et al., 2012), potential molecular mechanisms for this relationship are unclear. While emerging evidence is showing that osteocyte apoptosis plays a central role in mediating osteoclastic recruitment and local bone loss caused by fatigue bone damage (Verborgt et al., 2000), estrogen loss (Emerton et al., 2010) and aging (Jilka et al., 2014), whether this also applies to chemotherapy-induced bone defects has not been investigated previously. Thus, despite being poorly understood, apoptotic osteocytes have the potential to mediate the migration and differentiation of osteoclasts via intercellular communication (or signal crosstalk). Meanwhile, augmented expression in the bone of specific signaling molecules including a subset of pro-osteoclastogenic cytokines and chemokines (that induce migration of osteoclast precursor cells) was confirmed to play an important role in enhanced osteoclastogenesis (King et al., 2012; Wright et al., 2005; Yu et al., 2003a). However, whether these cytokines/chemokines

serve as an intercellular crosstalk signal(s) between osteocyte apoptosis and osteoclast recruitment/formation following chemotherapy has been unknown. Herein it is hypothesized that some cytokine(s) and/or chemokine(s) have the potential to act as a molecular link(s) between osteocyte apoptosis and osteoclast migration/differentiation after treatment, which results in the bone defects. Methotrexate (MTX) and dexamethasone (Dex), the two most commonly and intensively used agents in childhood oncology, particularly in the treatment of acute lymphoblastic leukemia (ALL) (Crofton et al., 2000) were chosen in the current study, and thus an *in vitro* MTX ± Dex treatment model in osteocytic cells was established to address this hypothesis.

Materials and Methods

Cell lines and culture conditions

IDG-SW3, an osteocytic cell line generated by crossing the immortomouse with the Dmp1-GFP transgenic mouse, was utilized in the current study as it was previously shown to be able to form mature osteocytes in osteogenic culture expressing osteocyte markers dentin matrix acidic phosphoprotein-1(DMP-1) and sclerostin (Woo et al., 2011). In brief, IDG-SW3 cells, (a generous gift from Dr Lynda Bonewald via Dr Gerald Atkins) were seeded at a density of 1 x10^6/flask for 2 days in T-75 culture flasks, which had been coated for 24 h with 3% rat tail type 1 collagen (C7661; Sigma, St Louis, MO), in proliferation medium containing alpha minimum essential medium (α-MEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 15 nM Hepes (PH7.4; Sigma), 100U/ml penicillin-100μg/ml streptomycin (Invitrogen) and 50 U/ml recombinant mouse interferon-γ (γ-INF; PMC4031; Invitrogen). Upon reaching confluence (day 0), the medium was replaced with the osteogenic differentiation medium, which is the proliferation medium additionally

supplemented with 50 μg/ml sodium ascorbate (Sigma), 4 mM β-glycerophosphate (Sigma) and in the absence of γ-INF. Cells were maintained in a humidified atmosphere (37˚C, 5% CO_2) and the medium was changed every two to three days. In addition, for time course cell differentiation gene expression studies, cells were also harvested at various time points (days 0, 4, 10 and 20) and stored at -80°C until RNA extraction.

MTX and/or Dex treatment and conditioned medium collection

To evaluate the potential changes induced by MTX ± Dex treatment, apart from osteogenic differentiation time-course studies, some IDG-SW3 cells were treated with MTX at 10^{-5}M and/or Dex at 10^{-6}M during the late stage of osteogenesis (day 20) (Woo et al., 2011) and harvested on day 22 after treatment for 48 hours. The untreated cells were considered as the control group. In addition, the conditioned medium was collected on day 20 before MTX ± Dex treatment. To collect MTX/Dex-free conditioned medium after treatment, the treated cells were rinsed with phosphate buffered saline (PBS) on day 22 and cultured for additional 24 h with fresh medium, after which conditioned medium was collected on day 23. The conditioned medium was also collected from untreated cells on days 20 and 22. The media were then spun down at 1500 RCF for 15 min and passed through a 0.22 μm filter (Sigma) to remove cellar debris. All conditioned media were stored at -20°C for further use.

Immunofluorescence analyses

It was demonstrated that, due to the DMP-1 driver function, green fluorescence protein (GFP) could be gradually expressed by IDG-SW3 cells during the progression of differentiating into mature osteocytes (Woo et al., 2011). Thus, after staining nuclei for 5 min with 0.5 μg/ml 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI; Sigma), the density of GFP-positive (GFP^+) cells was calculated as a percentage of DAPI-stained cells by using

fluorescence microscope to track the differentiation process. In addition, cleaved caspase-3, the active form of caspase-3, is well established as a marker for cell apoptosis (Budihardjo et al., 1999). Hence immunofluorescence analysis for cleaved caspase-3 was performed as described (Heshiki et al., 2015) to determine whether apoptosis was induced after MTX ± Dex treatment. In brief, all the cells were fixed with 4% paraformaldehyde in PBS for 15 min, and then permeabilized by ice-cold 90% methanol for 30 min. Cells were subsequently incubated with primary rabbit anti-cleaved-caspase-3 antibody (1:300; Asp175; Cell Signaling Technology, Beverly, MA) for 1 h at 37°C, followed by incubation with Alexa Fluor®-conjugated secondary antibody (1:500; Cell Signaling Technology) for 1 h. The nuclei were stained with 0.5 μg/ml DAPI (Sigma) for 5 min. The density of cleaved caspase-3 positive (cleaved caspase-3^{+}) cells was measured by using fluorescence microscope to score the apoptotic index.

Cytokine and chemokine PCR array

As a means to investigate the effects of MTX ± Dex treatment on the expression of the cytokines and chemokines, PCR array assays were carried out with RNA samples from MTX/Dex-treated or untreated IDG-SW3 osteocytic cells using a RT^2 Profiler PCR Array Kit for mouse cytokines & chemokines (PAMM-150ZD; QIAGEN Science, Germantown, MD) as instructed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was employed as the internal control gene. Any potential cytokine(s) and/or chemokine(s) which display prominent induction after treatment (e.g. CXCL12) were selected for further verification and investigation.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR was conducted to verify the altered expression levels of

cytokine(s)/chemokine(s) found by the PCR array studies above. In addition, qRT-PCR was also performed for some osteogenesis biomarkers (e.g. E11, Dmp-1, FGF-23 and SOST), osteoclastogenesis biomarker (e.g. cathepsin K) and some apoptosis-related molecules (e.g. Bcl-2, Bax, Fas, and Fas-L). All RNA samples were extracted from collected IDG-SW3 cells as described above. Total RNA was extracted using RNAqueous®-Micro Kit (Ambion; Applied Biosystems, Mulgrave, VIC, Australia) and single stranded cDNA was synthesized using iScript™ Reverse Transcription Supermix for RT-Qpcr kit (Bio-Rad, Hercules, CA). SYBR Green PCR assays (Bio-Rad) were then conducted in triplicate on cDNA samples. Primers were designed using NCBI Primer-Blast software (Tab.3-1) and obtained commercially (GeneWorks, Thebarton, SA, Australia). RT-PCR assays were carried out in CFX PCR System (Bio-Rad). Relative quantification was calculated with using the $2^{-\Delta}$CT method relative to the internal control gene Cyclophilin-A (Cyc-A).

Table 3-1. PCR primer pair sequences of each gene.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
E11	GTGGTGACTAGCCACTCTGT	ATTCCAACCAGGGTGACTAC
DMP-1	CTGAATCTGAAAGCTCTGAAG	TCCGTGTGGTCACTATTTGC
FGF-23	AGGTGAATGGGAGCAAGGTG	CTCCCTGTCGGTGGTGTTAG
SOST	CAACCAGACCATGAACCGGG	AAGCGGGTGTAGTGCAGCTC
Bcl-2	CAGCATGCGACCTCTGTTTG	TCTGCTGACCTCACTTGTGG
Bax	CGAGAGGTCTTCTTCCGTGT	GAGCACCAGTTTGCTAGCAA
Fas	AAGATCGATGAGATCGAGCACA	AAGCTTGACACGCACCAGTCT
Fas-L	GAGCTGTGGCTACCGGTGATAT	ACTCACGGAGTTCTGCCAGTTC
CXCL12	ATCAGTGACGGTAAGCCAGTCA	TGCACACTTGTCTGTTGTTGCT
Cathepsin K	GACTCAGAGTGCCACCTTCG	ACTGGCCCTGGTTTTTGACT
Cyc-A	CGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG

Enzyme-linked immunosorbent assay (ELISA)

To determine whether the observed alterations of cytokine and/or chemokine mRNA expression also persist in protein secretion, soluble protein concentrations of the selected cytokine(s) and/or chemokine(s) in conditioned medium were assessed by ELISAs with commercially available kits (R&D Systems, Minneapolis, MN) as described (Georgiou et al., 2012b).

Transwell migration assay

To investigate cytokine(s)/chemokine(s) contained in the conditioned medium from MTX and/or Dex-treated osteocytes affect osteoclast recruitment, an osteoclast precursor cell migration assay was carried out by using 24-well transwell plate with 8.0mm pore size polycarbonate membrane inserts (3422; Corning Costar Corp., Corning, NY). In brief, mouse RAW 264.7 osteoclast precursor cells were added to the upper chambers at 1×10^5 cells/well and the lower chambers were filled with conditioned medium from the treated or -untreated osteocytic cells. In addition, proliferation medium in the presence or absence of 10 nmol recombinant mouse CXCL12 protein (MAB350; R&D Systems) in 0.1% BSA/PBS was used in control groups as described (Yu et al., 2003b). Transwell units were incubated at 37°C in 5% CO_2 for 6 h and inserts were fixed with 10% formalin. Non-migrated cells on the upper side of the membranes were removed with a cotton-tipped applicator, whereas migrated cells on lower side of membranes were stained with 0.5% toluidine blue (Sigma). Then the migrated cells were counted under a light microscope. Meanwhile, the above migration assay in combination with blocking experiments was performed to further identify whether the cytokine/chemokine functional blockage has an influence on osteoclast precursor cell migration. Briefly, selected neutralizing antibodies such as 10μg/ml anti-CXCL12 antibody (MAB310; R&D Systems) were incubated with the conditioned medium for 2 h prior to

being added in the lower chambers, and the migration assay was carried out as described above (Zgraggen et al., 2014).

Osteoclast formation assay

The potential of the cytokine(s) and/or chemokine(s) produced by IDG-SW3 osteocytic cells following MTX and/or Dex treatment in promoting osteoclast formation was analyzed using an *in vitro* osteoclast formation assay. In brief, RAW264.7 osteoclast precursor cells were plated into 96-multiwell plates at $5x10^3$ cells/well and cultured in proliferation medium supplemented with 30 ng/ml receptor activator of nuclear factor-κB ligand (RANKL) (Peprotech, Rocky Hill, NJ), which was used as a osteoclastogenic stimulus, for 2 days. The medium was changed on day 3. Conditioned medium from MTX/Dex-treated and -untreated cells was added to the wells in a 1:1 ratio with fresh proliferation medium, and the concentration of RANKL was maintained at 30 ng/ml. In addition, both proliferation medium alone and the addition of 30 ng/ml RANKL (Peprotech) and/or 10 nmol recombinant mouse CXCL12 protein (MAB350; R&D Systems) were utilized in control groups. The culture was processed for 7 days and tartrate-resistant acid phosphatase (TRAP) staining was performed on day 9 (King et al., 2012). TRAP positive ($TRAP^+$) cells with three or more nuclei were identified as osteoclasts. Osteoclast formation was expressed as the density of multinucleated osteoclasts in each well (number/mm^2). In addition, to confirm the potential involvement of particular cytokine(s) and/or chemokine(s) in the conditioned medium in promoting osteoclast formation, selected neutralizing antibodies such as anti-CXCL12 antibody (MAB310; R&D Systems) were also utilized in blocking experiments as with the migration assay. The antibodies were incubated with the conditioned medium for 2 h and the osteoclast formation assay was subsequently performed in a similar fashion. Furthermore, some wells of RAW264.7 cells were collected to investigate the treatment effects on expression of

osteoclastogenesis-related marker gene Cathepsin K.

Statistical analysis

Statistical analysis was performed using Prims 6 software (GraphPad, San Diego, CA) and all results were expressed as means ± standard deviation (SD). One-way Analysis of Variance (ANOVA) and Tukey's post-hoc were used for time-course studies and testing the significant differences between MTX and/or Dex-treated and -untreated samples. A P-value < 0.05 is accepted as being statistically significant. P values of < 0.05, < 0.01, and < 0.001 were illustrated as *, ** and ***, respectively in the figures.

Results

Dynamics of the transition during IDG-SW3 cell osteogenic differentiation

To identify suitable culture time period for mature osteocytes to be treated by MTX, an IDG-SW3 osteocytic cell differentiation/maturation time-course study was firstly conducted. As IDG-SW3 cells have been illustrated to selectively express GFP during of the transition from osteoblasts to mature osteocytes, the maturation process could be monitored by Dmp1-GFP signal (Woo et al., 2011). In the current time-course study, there was a progressive and significant increase in the densities of GFP positive (GFP^+) cells from day 0 to day 20 ($P<0.01$, $P<0.001$ and $P<0.001$ on days 4, 10 and 20, respectively compared to day 0) (Fig.3-1A, 1B). To further evaluate IDG-SW3 differentiation *in vitro*, qRT-PCR for some typical osteogenesis biomarkers was performed. mRNA expression levels of E11 were found to be elevated on day 4 ($P<0.001$ compared to day 0) and return to baseline since day 10 ($P>0.05$) (Fig.3-1C). In addition, expressions of DMP-1 exhibited a gradual increase since day 4 (despite statistically insignificant compare to day 0), and reached a peak on day 20 ($P<0.01$)

(Fig.3-1D). A similar trend of increase was observed in FGF-23 expression ($P>0.05$, $P<0.05$ and $P<0.01$ on days 4, 10 and 20, respectively compared to day 0) (Fig.3-1E).

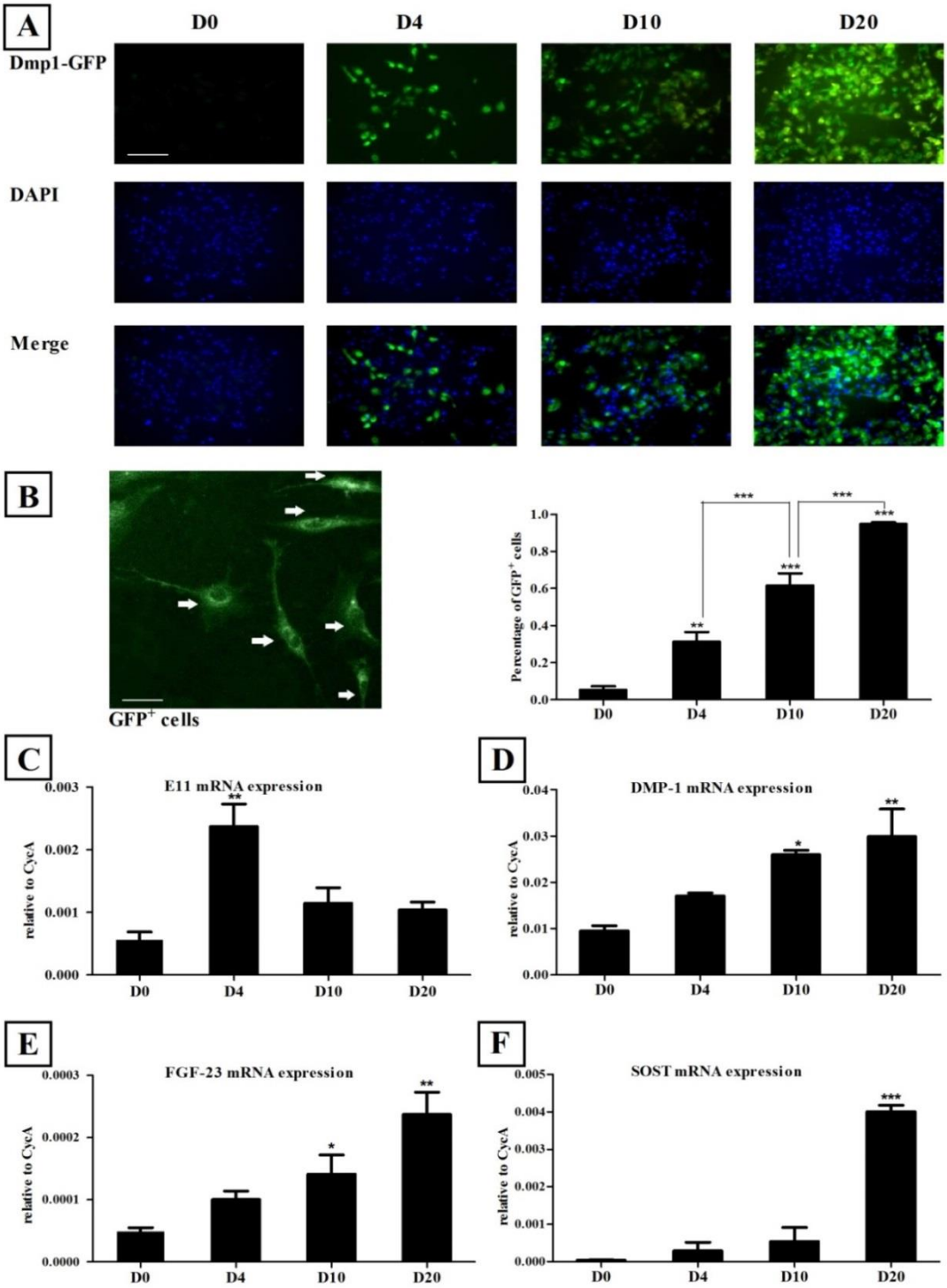


Figure 3-1. Observed alterations during IDG-SW3 differentiation in culture. (A) Representative images of DAPI staining in combination with Dmp1-GFP fluorometric analysis for IDG-SW3 cells during time-course study. **(B)** Comparison of the densities of GFP positive (GFP^+) cells among different time points. Quantitative RT-PCR relative gene analyses of osteogenesis and osteocyte-selective biomarkers relative to cyclophilin-A: **(C)**

E11, **(D)** DMP-1, **(E)** FGF-23 and **(F)** SOST**.** Scale bar on panels A, B = 400 and 50 μm, respectively.

Meanwhile, although there were no significant differences in expression of SOST on days 4 and 10, an obvious increase were examined on day 20 ($P<0.001$ compared to day 0). These results (abundant expression of osteocyte maturation markers DMP1, FGF23, and SOST) suggested that the IDG-SW3 cells reached mature osteocytes after culture for 20 days.

Osteocyte apoptosis induced by MTX and/or Dex treatment

To evaluate apoptosis in IDG-SW3 osteocytes following MTX ± Dex treatment *in vitro*, IDG-SW3 cells were treated with MTX at 10^{-5}M and/or Dex at 10^{-6}M starting from day 20 (during the mature stage as identified in the above time course study) and harvested on day 22 after 48 h treatment. Since cleaved caspase-3 has been implicated as a key mediator of apoptosis (Budihardjo et al., 1999), immunofluorescent double labeling of IDG-SW3 cells was carried out to calculate the density of cleaved caspase-3^+ cells, and to further evaluate apoptosis after treatment (Fig.3-2A). It was observed a considerably increase of cleaved caspase-3^+ cell density in MTX ± Dex-treated osteocytic cells in comparison to the -untreated cells ($P<0.001$) (Fig.3-2B).

Aside from immunofluorescence analysis, real-time qRT-PCR was performed to examine the changes in expression levels of apoptosis regulatory genes (e.g. Bax, Bcl-2, Fas and Fas-L). The expression levels of pro-apoptotic protein Bax were significantly enhanced in each treatment group ($P<0.001$) (Fig.3-2C). While no significant differences in expression of anti-apoptotic protein Bcl-2 were examined following Dex alone and Dex + MTX combination

treatment, the expression levels of Bax were obviously decreased after MTX treatment ($P<0.05$) (Fig.3-2D) and the Bax/Bcl-2 ratio achieved a significant increase among three treatment groups ($P<0.001$) (Fig.3-2E).

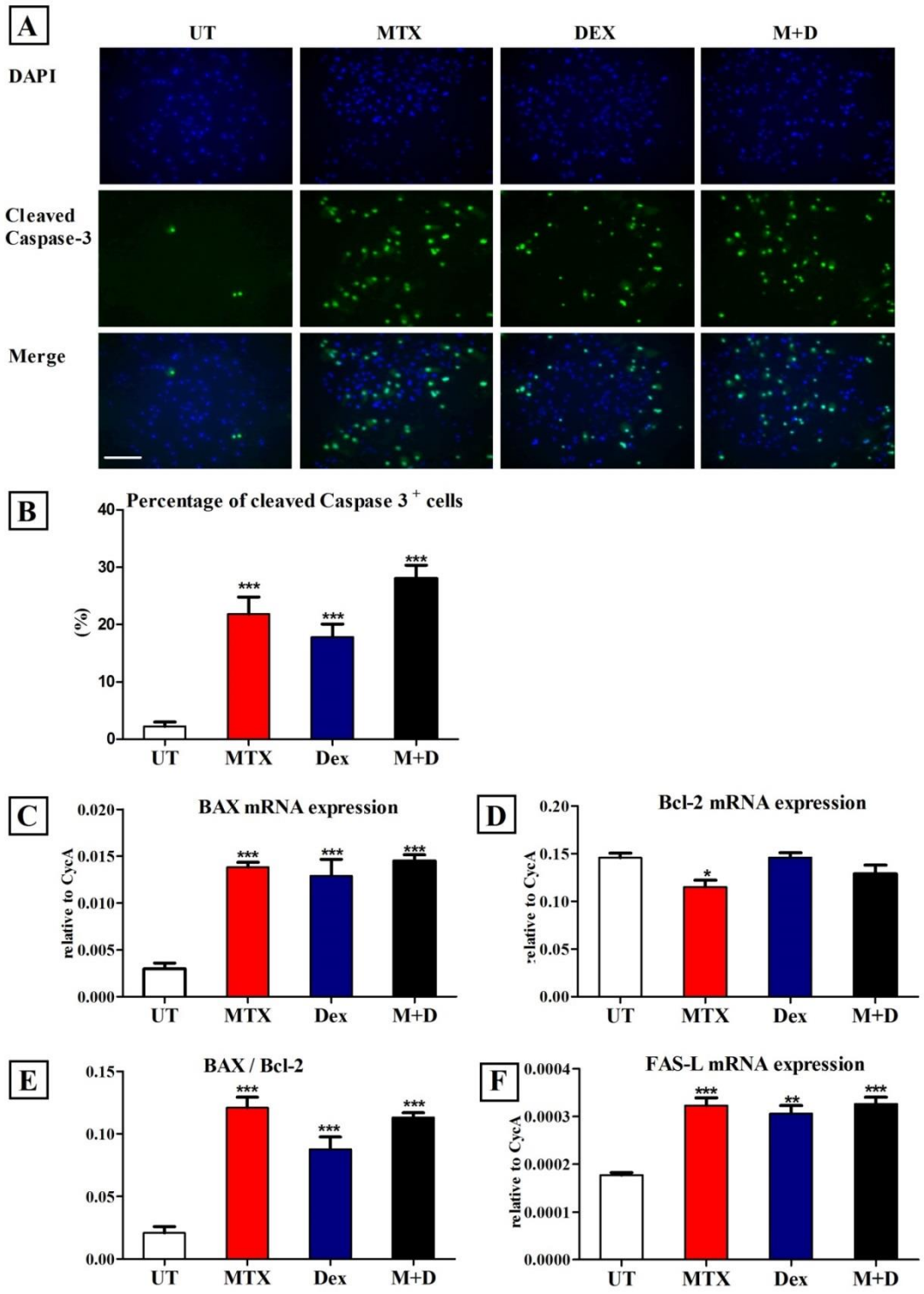


Figure 3-2. Effects of MTX treatment on osteocyte apoptosis. (A) Representative images of immunofluorescent double labeling for IDG-SW3 osteocytic cells in 10^{-5}M MTX-treated (MTX), 10^{-6}M Dex-treated (Dex), 10^{-5}M MTX + 10^{-6}M Dex MTX combination-treated

(M+D), and -untreated (UT) groups *in vitro*. Cleaved Capase-3^+ positive staining and nuclear marker DAPI are labeled with green and blue, respectively. **(B)** Percentage of cleaved Capase-3^+ cells. Treatment effects on mRNA expression of apoptosis regulatory genes (relative to Cyclophilin-A or CycA): **(C)** Bax, (**D)** Bcl-2, **(E)** Bax/Bcl-2 ratio, and **(F)** Fas-L. Scale bar on panel A = 400 μm.

Meanwhile, although only low levels and no obvious changes of Fas could be detected flowing treatment, the expression level of Fas ligand (Fas-L) displayed a noticeable up-regulation by MTX ± Dex treatment ($P<0.001$) (Fig.3-2F).

Augmented cytokine and chemokine expression levels following MTX and/or Dex treatment

As a means of examining the effects of MTX ± Dex treatment on cytokine/chemokine expression, a particular PCR array was performed. It was shown that the expression levels of a number of cytokines (e.g. TNF-α, IL-2a, -6, and -17f) and chemokines (e.g.CXCL12, CCL2, CCL5, and CXCL1) were noticeably upregulated after treatment (Fig.3-3). In particular, CXCL12 was identified to achieve the most prominent induction in each treatment group and herein selected for further examination.

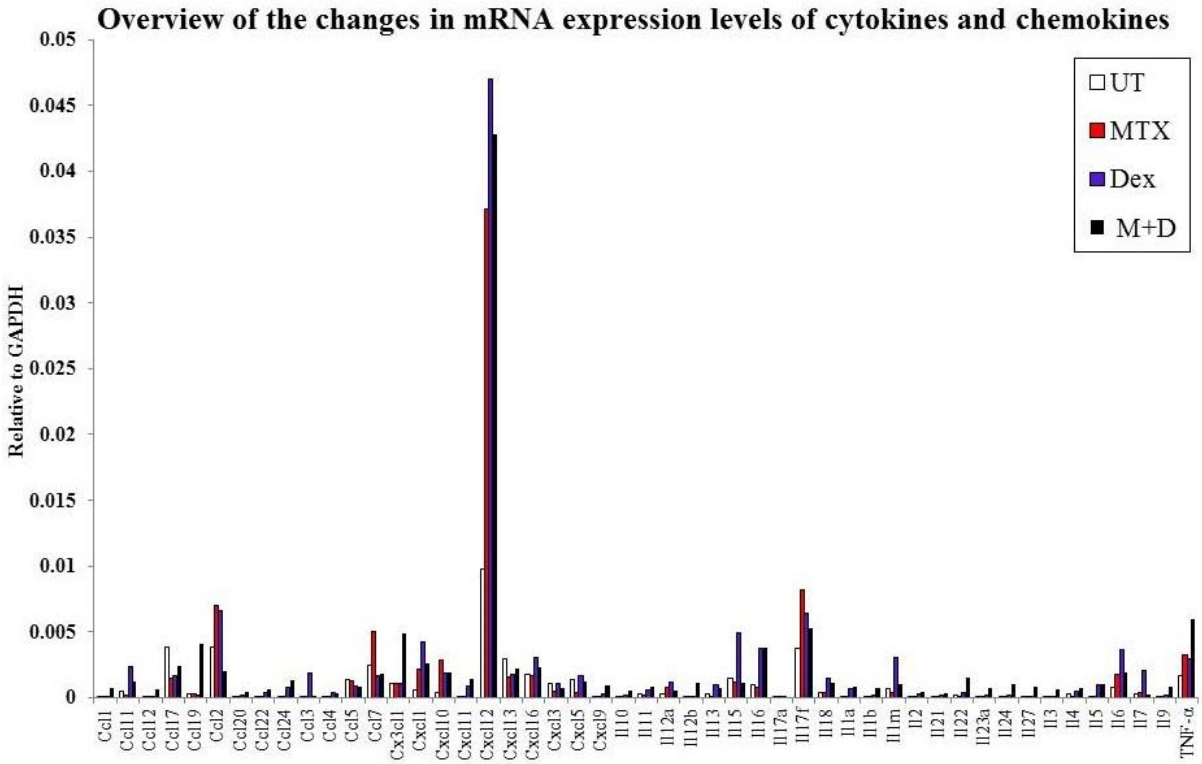


Figure 3-3. Induction of cytokine and chemokine expression in IDG-SW3 cells following MTX and/or Dex treatment. Overview of the expression levels of the major known cytokines and chemokines. CXCL12 achieved the most prominent induction after 10^{-5}M MTX (MTX), 10^{-6}M Dex (Dex), 10^{-5}M MTX + 10^{-6}M Dex (M+D) combination treatment when compared to the untreated (UT) control.

Prominent induction of CXCL12

Quantitative RT-PCR and ELISA were conducted to confirm the augmented expression of CXCL12 observed by the PCR array. Using the samples from MTX and/or Dex-treated and -untreated IDG-SW3 osteocytic cells, both mRNA expression level ($P<0.001$) (Fig.3-4A) and protein concentration in the conditioned medium ($P<0.05$, $P<0.05$ and $P<0.01$ after 10^{-5}M MTX, 10^{-6}M Dex, 10^{-5}M MTX + 10^{-6}M Dex combination treatment, respectively compared to untreated control) (Fig.3-4B) were found to be significantly increased following MTX ± Dex treatment *in vitro*.

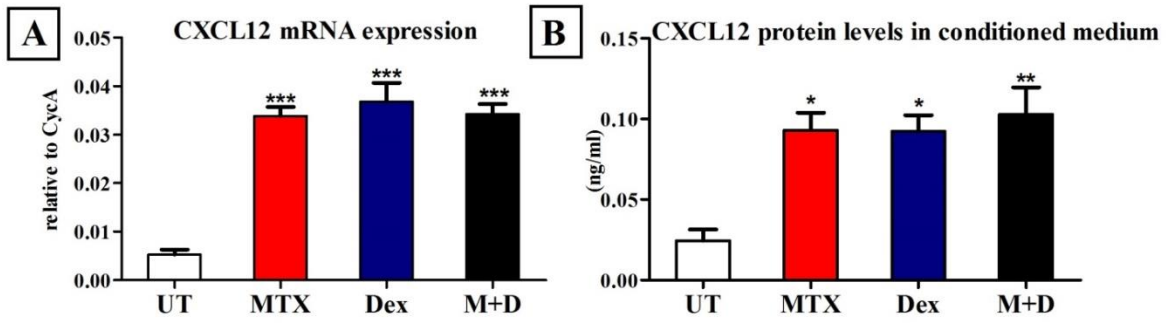


Figure 3-4. Effects of MTX and/or Dex Treatment on CXCL12 expression. (A) Quantitative RT-PCR analysis for CXCL12 mRNA expression in 10^{-5}M MTX (MTX), 10^{-6}M Dex (Dex), 10^{-5}M MTX + 10^{-6}M Dex (M+D) combination-treated, and -untreated (UT) IDG-SW3 cells *in vitro*. (B) CXCL12 protein levels (ng⁄ml) as determined by ELISA in conditioned medium with/without MTX ± Dex treatment.

Stimulated chemotaxis of osteoclast precursors by CXCL12 in conditioned medium of MTX and/or Dex-treated IDG-SW3 osteocytic cells

In order to examine the potential function in osteoclast recruitment CXCL12 of which was induced and contained in the conditioned medium of MTX ± Dex-treated osteocytic cells, osteoclast precursor RAW264.7 cells were subjected to an *in vitro* migration assay as promoted by the conditioned medium with and without CXCL12 neutralizing antibody blockade (Fig.3-5). It was observed that cell migration was induced in the presence of 10 nmol exogenous recombinant CXCL12 protein ($P<0.05$). Compared with the conditioned medium from -untreated cells, the conditioned medium from MTX ± Dex-treated cells, which has a higher content of CXCL12 could considerably promote osteoclast precursor cell migration ($P<0.05$, $P<0.01$ and $P<0.05$ in MTX, Dex, MTX + Dex combination treatment groups, respectively compared to untreated control). However, this migration promotion response was significantly repressed by the neutralizing anti-CXCL12 antibody among all the treatment groups ($P<0.05$).

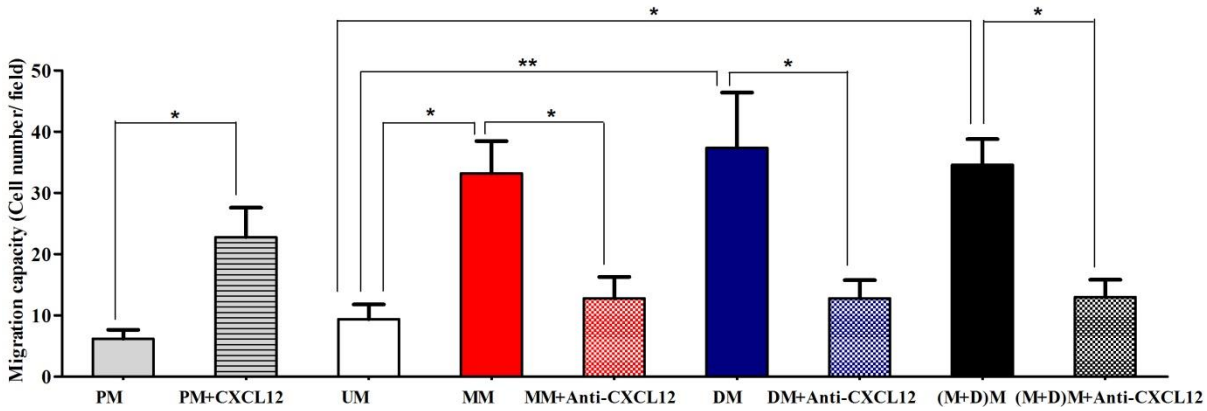


Figure 3-5. Potential functional effect of CXCL12 contained in the conditioned medium from MTX ± Dex-treated IDG-SW3 cells in stimulating osteoclast precursor cell migration. *In vitro* RAW264.7 osteoclast precursor migration assay was carried out and applied with conditioned medium of MTX and/or Dex -untreated or -treated IDG-SW3 osteocytic cells with or without an anti-CXCL12 neutralising antibody. The densities of migrated cells were determined: proliferation medium (PM) alone; PM supplemented with 10 nmol recombinant CXCL12 protein (PM+CXCL12); conditioned medium from -untreated IDG-SW3 cells (UM); conditioned medium from MTX (10^{-5}M)-treated cells (MM), MM additionally with 10μg/ml neutralizing anti-CXCL12 antibody; conditioned medium from Dex (10^{-6}M)-treated cells (DM); DM additionally with 10μg/ml neutralizing anti-CXCL12 antibody; conditioned medium from MTX (10^{-5}M) + Dex (10^{-6}M) combination-treated cells ((M+D)M); and (M+D)M supplemented with 10μg/ml neutralizing anti-CXCL12 antibody.

Osteoclast formation enhanced by CXCL12 contained in the conditioned medium of MTX and/or Dex-treated osteocytic cells

Osteoclast precursor RAW264.7 cells applied with conditioned medium from MTX ± Dex-treated or -untreated osteocytic cells were also subjected to an *in vitro* osteoclast formation assay (Fig.3-6). Similarly, both 10 nmol recombinant mouse CXCL12 protein (MAB350; R&D Systems) and 10 μg/ml neutralizing anti-CXCL12 antibody (MAB310; R&D Systems) were utilized in the study. It was found that RANKL is mandatory for osteoclast differentiation (Fig.3-6A, B) and exogenous recombinant CXCL12 protein had no capacity to induce osteoclast formation in the absence of RANKL (Fig.3-6C, D).

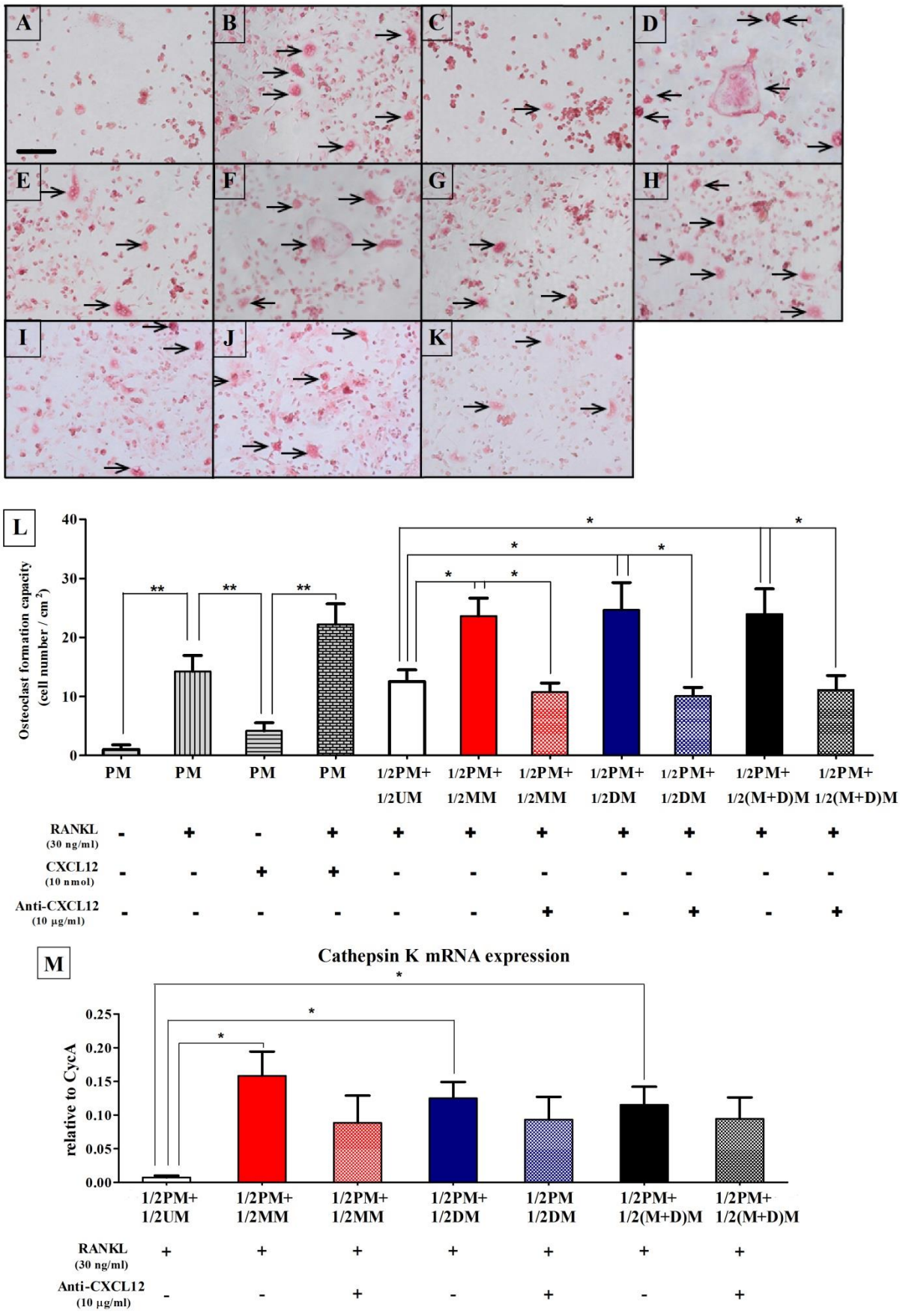


Figure 3-6. Potential of CXCL12 contained in MTX ± Dex-treated IDG-SW3 cell conditioned medium in promoting osteoclast formation. *In vitro* osteoclast formation

assay was carried out with RAW264.7 cells incubated with different media: **(A)** proliferation medium (PM) alone; **(B)** PM supplemented with 30ng/ml RANKL; **(C)** PM supplemented with 10 nmol recombinant CXCL12 protein (PM+CXCL12); (**D)** PM+CXCL12 in combination with 30ng/ml RANKL; (**E)** Mixture of MTX-untreated cell conditioned medium (UM) with fresh PM (1/2UM+1/2PM) supplemented with 30 ng/ml RANKL; (**F)** Mixture of MTX(10^{-5}M)-treated cell conditioned medium (MM) with fresh PM (1/2MM+1/2PM) supplemented with 30 ng/ml RANKL; (**G)** (1/2MM+1/2PM) additionally with 10 μg/ml neutralizing anti-CXCL12 antibody and 30 ng/ml RANKL; (**H)** Mixture of Dex (10^{-6}M)-treated cell conditioned medium (DM) with fresh PM (1/2MM+1/2PM) supplemented with 30 ng/ml RANKL; **(I)** (1/2DM+1/2PM) additionally with 10 μg/ml neutralizing anti-CXCL12 antibody and 30 ng/ml RANKL; **(J)** Mixture of MTX (10^{-5}M) + Dex (10^{-6}M) combination -treated cell conditioned medium ((M+D)M) with fresh PM (1/2(M+D)M +1/2PM) supplemented with 30 ng/ml RANKL; and **(K)** (1/2(M+D)M +1/2PM) additionally with 10 μg/ml neutralizing anti-CXCL12 antibody and 30 ng/ml RANKL. Osteoclasts were detected by TRAP staining and cells with three nuclei depicted by black arrows (**A-K**). **(L)** The densities of multinucleated $TRAP^+$ osteoclasts were determined. **(M)** Quantitative RT-PCR relative gene expression analyses for cathepsin K among RAW264.7 cells incubated with conditioned media from MTX ± Dex treated/untreated IDG-SW3 cells. Scale bar on panel A = 400 μm (also applies on panels B-K).

Interestingly, companied to RANKL, MTX ± Dex-treated cell conditioned medium was observed to be capable of significantly promoting osteoclast formation in comparison to conditioned medium from -untreated cells ($P<0.05$) (Fig.3-6E, F, H, J, L). In parallel, the mRNA expression levels of osteoclastogenesis marker cathepsin K were noticeably increased in RAW264.7 cells incubated with conditioned medium from MTX ± Dex-treated IDG-SW3 cells ($P<0.05$) (Fig.3-6M). On the other hand, consistent with the findings in the migration assay, this stimulating effect was also considerably repressed by the presence of the CXCL12 neutralizing antibody ($P<0.05$) (Fig.3-6G, I, K, L).

Discussion

Despite unclear mechanisms, intensive chemotherapy has been shown to significantly disrupt the steady-state cell regulation in bone and bone marrow microenvironment and result in the severe bone loss. Based on previous studies, it is proposed that some signalling molecules (e.g. cytokines/chemokines) derived from apoptotic osteocytes have the potential role in

inducing osteoclast precursor cell recruitment and differentiation after MTX and/or Dex treatment, leading to the increased bone resorption. Using a cultured osteocyte MTX ± Dex treatment model, the current study observed that MTX and/or Dex induces osteocyte apoptosis and upregulation of some cytokines and chemokines (with particular CXCL12 being shown to have the most obvious induction) *in vitro*. In addition, apoptotic osteocyte-derived CXCL12 (contained in conditioned medium from MTX ± Dex-treated osteocytes) was found to significantly stimulate osteoclast migration and formation as the promoting effects could be suppressed by the presence of a neutralizing anti- CXCL12 antibody.

An ideal in vitro model to produce mature osteocytes for the MTX and/or Dex treatment studies

Osteocytes are derived from osteoblast lineage cells which are progressively embedded in the mineralized matrix (Schaffler and Kennedy, 2012). Although osteocytes are the most abundant cellular component of bone tissue, their locations within the surrounding bone matrix makes *in vivo* study technically challenging. Meanwhile, since osteocytes are the terminally differentiated cells, *in vitro* studies for primary osteocytes are also limited due to the inability to proliferate (Stern et al., 2012). Recently, IDG-SW3, an immortomouse/Dmp1-GFP-derived osteoblastic/osteocytic cell line in which GFP expressions is variable in osteoblast and osteocyte phenotypes, has been well characterized and widely used for osteocyte differentiation research (Woo et al., 2011). Thus, an *in vitro* IDG-SW3 model system was developed and utilized in the current study. As expected, our time course study observed sustainable and significant increases in the densities of GFP^+ cells during osteocyte differentiation/maturation, indicating the transition toward osteocytic phenotypes.

Meanwhile, these phenotypic changes were confirmed by progressive changes in levels of

gene expression for specific osteogenesis and osteocyte-selective biomarkers as observed by qRT-PCR analyses. E11, also known as GP38, is a membrane-bound protein expressed by the differentiating osteocytes and is considered as the earliest osteocyte marker (Dallas et al., 2013). Sclerostin, a secreted glycoprotein encoded by the SOST gene, is observed to be highly expressed by terminally differentiated cells deeply embedded in mineralized matrix and thus considered as a biomarker for mature osteocytes (Poole et al., 2005). In current study, it was found that E11 expression level peaked on day 4 and returned to baseline since day 10, while SOST expression maintained in low but detectable levels in earlier time points but dramatically increased on day 20. These findings suggest IDG-SW3 cells may differentiate into early osteocytes from day 4 as well as mature osteocytes from day 20. In addition, both DMP-1 and fibroblast growth factor-23 (FGF-23) are demonstrated to act as osteocyte biomarkers. Osteocytes have been confirmed to selectively express DMP-1 to develop and maintain bone matrix in the mineralization process (Jacobs et al., 2010; Kramer et al., 2010). FGF-23, predominantly produced by osteocytes, has been revealed to be vital to the coordination of inorganic mineral transportation of the bone-kidney axis (Crofton et al., 2000). The current study has also observed a similar upward trend in expression of DMP-1 and FGF-23 during the differentiation period between day 0 and day 20, which recapitulates the differentiation process from late osteoblasts to mature osteocytes. Above all, the current study has confirmed the suitability of IDG-SW3 cell line as a model for forming mature osteocytes and has identified the day 20 of osteogenic culture being a suitable time point for our MTX ± Dex treatment studies with cultured mature osteocytes.

MTX and/or Dex treatment significantly induced osteocyte apoptosis

It has been stated that osteocytes are with long lifespan and only about 2.5% osteocytes undergo apoptosis each year under physiologic conditions (Manolagas and Parfitt, 2010).

Although the normal rate of osteocyte apoptosis is confirmed to be vital for skeletal health (Jilka et al., 2014), emerging evidence has revealed that external influences such as aging, pausimenia, under-loading or unloading, microdamage and pathological conditions are responsible for the induction of osteocyte apoptosis, leading to the increased skeletal fragility (Almeida et al., 2007; Jilka et al., 2007; Weinstein and Manolagas, 2000). In particular, previous *in vivo* studies have shown that intensive use of some chemotherapeutic agents (e.g. MTX and Dex) could considerably promote osteocyte apoptosis, which is associated with the bone defects (Spreafico et al., 2008; Weinstein, 2011; Xian et al., 2008; Xian et al., 2007). In the current study, IDG-SW3 osteocytic cells were treated with MTX and/or Dex in the mature osteocyte stage *in vitro*. Significantly enhanced apoptosis induction was observed by immunofluorescence analysis for cleaved caspase-3, a central marker for apoptosis, in response to MTX ± Dex treatment.

In addition, it has been well established that apoptosis is mainly mediated by two major signaling pathways in mammalian cells: the intrinsic and extrinsic pathways, which are determined by regulatory genes Bax/Bcl-2 and Fas/Fas-L respectively (Danial and Korsmeyer, 2004). In the current study, real-time qRT-PCR analyses of mature osteocytic cells showed that MTX and/or Dex treatment significantly increased the ratio of expression levels of pro-apoptotic Bax over anti-apoptotic Bcl-2, as well as increased the expression level of another apoptosis inducer Fas-L despite no obvious changes in death receptor Fas. Interestingly, the expression levels of Bax and Bcl were simultaneously enhanced after Dex treatment. Similar to the findings in the present study, upregulated expression levels for both BAX and Bcl-2 were observed after fatigue loading studies *in vivo*. These results suggest that Bax/Bcl-2 play a complimentary role in osteocyte apoptosis (Manolagas and Parfitt, 2010). In particular, they were expressed in fundamentally different patterns and specific spatial

distributions of Bax and Bcl-2 in osteocyte apoptosis were revealed: the pro-apoptotic protein Bax was highest adjacent to the damage focus, whereby the anti-apoptotic Bcl-2 had the prominent expression more distant from the microcracks. Nonetheless, the current findings indicate that MTX ± Dex-induced osteocyte apoptosis may be briefly regulated through both the intrinsic apoptotic pathway and the extrinsic Fas-L/Fas pathway in the current *in vitro* model. However, further studies are still required to investigate the mechanisms involved in osteocyte apoptosis after chemotherapy.

Conditioned medium of MTX and/or Dex-treated osteocytic cells significantly stimulated osteoclast precursor migration and formation

While the process of bone resorption involves sequential stages such as osteoclast precursor cells migration and the following differentiation of osteoclast precursors into multinucleated and activated osteoclasts (Kikuta and Ishii, 2013), it is not clear how osteoclast precursors are selectively recruited thereafter develop into resorbing osteoclasts. Previously, some studies have suggested that osteocyte apoptosis may play an important role in mediating osteoclastic recruitment and local bone loss in situations such as fatigue bone damage, oestrogen loss and aging (Emerton et al., 2010; Jilka et al., 2014; Verborgt et al., 2000). In an effort to address the potential role of osteocyte apoptosis caused by MTX and/or Dex treatment in osteoclast recruitment, the current study treated osteoclast precursor cells with conditioned medium from MTX ± Dex-treated vs -untreated osteocytic cells, and found that conditioned medium from treated osteocytes has the capacity to considerably enhance osteoclast precursor cell migration and osteoclast formation, suggesting some potent osteoclastogenesis-stimulating factors have been derived from apoptotic osteocytes after MTX and/or Dex treatment in the present *in vitro* model.

MTX and/or Dex treatment induced osteocyte apoptosis and enhanced osteoclast precursor migration and formation, which is molecularly linked by the CXCL12

While intercellular communication is generally well-characterized to be crucial for skeletal development and maintenance, it is evident that osteocytes exert direct effects on osteoclastogenesis and bone remodeling (Nakashima et al., 2011; O'Brien et al., 2013; Xiong et al., 2011). Recent studies also found that induction of osteocyte apoptosis tightly correlates with promoted osteoclast recruitment and differentiation as well as enhanced bone resorption (Bellido, 2014; Kennedy et al., 2012; Plotkin, 2014). In particular, a specific temporal-spatial association between the accumulation of apoptotic osteocytes and the increased presence of osteoclasts following chemotherapy has been illustrated (Shandala et al., 2012). Although the underlying mechanisms still remain largely unknown, these findings suggest that apoptotic osteocytes have the potential to express some regulatory factors to coordinate osteoclast formation and activity after treatment. Cytokines and chemokines are demonstrated to be key mediators in trafficking, differentiation, and activity of skeletal cells under physiological and pathological condition (Caetano-Lopes et al., 2009; Edderkaoui, 2017; Lee et al., 2008). Especially, augmented expression of specific cytokines/chemokines has been found to play an important role in enhanced osteoclastogenesis (King et al., 2012; Shahnazari et al., 2013; Wright et al., 2005; Yu et al., 2003a). In the current study, the expression of 84 common cytokines and chemokines was systematically analysed by using a PCR array following MTX and/or Dex treatment. It was observed that, the expression levels of some cytokines (e.g. IL-17f and TNF-α) and chemokines (e.g. CXCL12, CCL2 and CCL5) in osteocytes were significantly upregulated after treatment by MTX, Dex, or their combination. In particular, chemokine CXCL12, a member of the CXC family of chemokines, was found to exhibit the most prominent induction in each treatment group. In addition, the upregulation of CXCL12 in MTX ± Dex-treated osteocytic cells was confirmed by subsequent studies which showed

the significant increase in mRNA expression level in apoptotic osteocytes and the protein concentration in the conditioned medium. These findings suggest that CXCL12 could be a major molecule produced by apoptotic osteocytes after MTX and/or Dex treatment which could be involved in causing the increased osteoclast recruitment and bone loss observed.

Since previous studies have shown that CXCL12 plays an important role in osteoclast precursor cell recruitment and development as well as bone resorption (Diamond et al., 2009; Gronthos and Zannettino, 2007; Wright et al., 2005; Yellowley, 2013; Yu et al., 2003a; Zannettino et al., 2005), the current study subsequently investigate the potential role of apoptotic osteocyte-derived CXCL12 in osteoclast precursor cell migration and osteoclast formation *in vitro.* It was observed that exogenous recombinant CXCL12 protein as well as the conditioned medium (containing 0.093±0.011, 0.092±0.009, 0.102±0.017 ng/ml CXCL12 protein, respectively) from MTX, Dex, or their combination-treated osteocytes (but not the untreated cells) were capable of inducing osteoclast migration, which were blocked dose-dependently by the neutralizing anti-CXCL12 antibody. These findings suggest that CXCL12 is an effective chemoattractant for osteoclast precursor cells and that CXCL12 derived from apoptotic osteocytes following treatment can indeed promote osteoclast precursor cell recruitment. The current study has also investigated a potential role of apoptotic osteocyte-derived CXCL12 in osteoclast formation *in vitro*. Interestingly, while CXCL12 alone (in the absence of RANKL) could support osteoclast formation to a low degree, in the presence of RANKL, recombinant CXCL12 protein as well as CXCL12 contained within the MTX, Dex, or their combination-treated cell conditioned medium could significantly stimulate osteoclast formation compared to the conditioned medium from the untreated cells, and to the extents of 156.3%, 166.2%, 173.6% and 168.7% higher, respectively, than the presence of RANKL alone. Furthermore, this promoting effect of the treated cell conditioned medium could be

considerably inhibited by neutralization of CXCL12 by an anti-CXCL12 antibody (to the level exerted by the untreated cell condition medium). The above findings suggested that apoptotic osteocyte-derived CXCL12 can be an important osteoclast recruitment and stimulating factor due to its direct and/or indirect effects on osteoclast precursor cell migration and formation, and thus has the potential to act as a molecular linker between osteocyte apoptosis and osteoclast precursor recruitment and differentiation after MTX and/or Dex treatment.

Interestingly, elevated serum CXCL12 levels were observed (Georgiou et al., 2012b), which showed a temporal relationship with the increased osteoclastic recruitment and resorption in rats after MTX chemotherapy (King et al., 2012). While upregulated serum levels of CXCL12 are associated with increased osteoclast activity and osteolysis in multiple myeloma patients (Zannettino et al., 2005), further studies are required to investigate the roles of apoptotic osteocyte-derived CXCL12 or its higher circulation level in bone loss following chemotherapy. Further work is also required to clarify the action mechanisms of apoptotic osteocyte-derived CXCL12 in the cell crosstalk for osteoclast recruitment and formation (including function of CXCL12 receptor and its downstream signal pathway). In addition, while CXCL12 has been found to be the major chemokine and perhaps a major player secreted by apoptotic osteocytes after treatment, future work is also required to investigate potential roles of other molecules induced in the apoptotic osteocytes including osteoclastogenic factors (RANKL, IL-6, TNF-α) in the osteoclastic bone loss following chemotherapy.

Conclusion

The current study has demonstrated that CXCL12 is a novel osteoclast stimulating factor derived from apoptotic osteocytes following MTX and/or Dex treatment, which can promote osteoclast recruitment that is known to lead to aggravated bone resorption and bone loss. Our findings are exciting as an emerging role has been highlighted for CXCL12 in promoting osteoclast precursor migration to resorption sites, their fusion into osteoclasts and in pathological bone loss. Since pathological bone loss can be inhibited by blocking the CXCL12/CXCR4 signaling (Diamond et al., 2009), the current study can provide a molecular basis for future therapeutic approaches for preventing chemotherapy-induced bone loss including the use of CXCL12 antibodies and/or receptor inhibitors which will be investigated in future studies.

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Disclosure

The authors declared that they have no conflict of interests to this work.

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CHAPTER FOUR

Study 3: Role of CXCL12 in the development of bone growth defects following dexamethasone therapy: a potential novel therapeutic target

Statement of Authorship

Role of CXCL12 in the development of bone growth defects following dexamethasone therapy: a potential novel therapeutic target

Qian Tang (Candidate)
Planned and carried out all the relevant experiments in project. Performed concept, prepared figures, drafted, edited and revised the manuscript. The current manuscript is prepared for submission to *J Bone Miner Re.*
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: ……21/09/17……

Chia-Ming Fan
Participated in animal trials, tissue collection, *ex vivo* osteoclast formation assay.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: ……21/09/17……

Rosa Chung
Participated in animal trials, tissue collection, *ex vivo* osteoclast formation assay.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]…… Date: ……21/09/17……

Yu-Wen Su
Participated in animal trials and immunohistochemical analyses.
I hereby certify that the statement of contribution is accurate.

Signed: ……[redacted]… Date: ……21/09/17……

Cory J. Xian
Contributed to conception of the project, supervision of project development, data interpretation, and critical manuscript evaluation and acted as the corresponding author.
I hereby certify that the statement of contribution is accurate.

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Role of CXCL12 in the development of bone growth defects following dexamethasone therapy: a potential novel therapeutic target

Qian Tang, Chia-Ming Fan, Rosa Chung, Yu-Wen Su, Cory J. Xian

Sansom Institute for Health Research, School of Pharmacy and Medical Sciences, University of South Australia, Adelaide, SA 5001, Australia;

* Corresponding author: Cory J Xian, PhD

School of Pharmacy and Medical Sciences

University of South Australia

City East Campus, GPO Box 2471

Adelaide 5001, Australia

Tel: +61 8 830 21944

Fax: +61 8 830 22389

Email: Cory.Xian@unisa.edu.au

Abstract

Intensive dexamethasone (Dex) therapy is known to cause bone growth defects for which the mechanisms remain unclear and there are currently no effective treatments. Since chemokine CXCL12 has been identified in our recent studies to have the most prominent induction (among all chemokines) in Dex-induced apoptotic chondrocytes/osteocytes and CXCL12 derived from these apoptotic cells can promote osteoclast precursor cell migration and osteoclast formation *in vitro*, the current study investigated the potential effects of chemokine CXCL12 blockade in preventing bone defects in rats caused by Dex treatment. Male rats of 6-week-old were orally gavaged daily with saline or Dex at 1 mg/kg/day for consecutive 14 days, and received intraperitoneal injection of anti-CXCL12 neutralizing antibody or normal mouse IgG at 1 mg/kg (3 times per week) during 2-week Dex administration. Dex treatment was shown to cause an obvious reduction in growth plate thickness, primary spongiosa height, trabecular bone volume, which are associated with induced chondrocyte/osteocyte apoptosis, enhanced chondroclast/osteoclast recruitment, stimulated osteoclastogenic differentiation potential, marginally inhibited osteoblastogenic differentiation potential and promoted marrow adipogenic differentiation potential. CXCL12 was found to be obviously induced in apoptotic growth plate chondrocytes and metaphyseal bone osteocytes. Blocking CXCL12 function by the supplementation of the anti-CXCL12 neutralizing antibody was observed to significantly prevent Dex-induced growth plate cartilage and bone loss. Thus, this study has confirmed *in vivo* the role of CXCL12 as a molecular link between Dex treatment-induced skeletal cell apoptosis and chondroclastic/osteoclastic recruitment and growth plate cartilage/bone loss, and has revealed a therapeutic potential of blocking CXCL12 function in preventing bone growth defects during/after Dex treatment.

Key Words

Glucocorticoid, Bone growth defects, CXCL12, Chondrocytes, Osteocytes, Chondroclasts, Osteoclasts, Adipocytes

Introduction

Dexamethasone (Dex) is a potent member of the glucocorticoid class of steroids which has strong anti-inflammatory and immunosuppressive properties. While Dex has become one of the most commonly and intensively used therapeutic agents in clinical practice, particularly in paediatric oncology, and has achieved increasing success (Crofton et al., 2000), Dex has been revealed to exert opposing effects on various aspects of regulation pathways of human body systems including the skeletal and immune systems. In particular, it has been shown that intensifying use of Dex therapy is associated with severe bone growth defects such as bone growth arrest, bone loss, osteoporosis and associated fractures in the long term (Canalis et al., 2007). Although the underlying mechanisms still remain unclear, it is evident that these defects are related to the alterations induced by Dex treatment in multiple skeletal cell regulation pathways within the bone and marrow microenvironment (Xian et al., 2007). Characteristic disruption of skeletal cell regulations such as induced chondrocyte/osteocyte apoptosis accompanied by enhanced chondroclast/osteoclast recruitment and activity have been observed following Dex treatment, which results in uncontrolled cartilage and/or bone loss and the impairment of bone growth (Fan et al., 2011; Olney, 2009; Zaman et al., 2012). However, whether these Dex-induced disruptions to steady-state skeletal cellular homeostasis occur dependently or independently of one another still requires further investigation. More importantly, due to the lack of preventive strategies and specific treatments to minimizing Dex therapy-associated adverse skeletal effects, it has become more urgent to understand the pathogenesis of these defects and to develop treatments.

Recently, a similar coupling has been observed between the accumulation of apoptotic chondrocytes/osteocytes and the increase in recruitment of chondroclast/osteoclasts as driven by various external factors (e.g. chemotherapeutic agent treatment) has been observed

(Bellido, 2014; Fan et al., 2012; Kennedy et al., 2012; Plotkin, 2014; Shandala et al., 2012), suggesting an underlying intercellular crosstalk which tightly coordinates skeletal cell regulations in response to external influences. In addition, the interplay between skeletal and immune systems has been gradually highlighted. Accumulating evidence has revealed their overlapping regulatory mechanisms by which skeletal and immune cells shared the regulators in common, including specific chemokine(s) (Caetano-Lopes et al., 2009). Chemokines are a large family of small chemotactic cytokines and well-known to regulate the migration and positioning of immune cells (Su et al., 2017). Although chemokines are originally identified as the mediators of immune response, a subset of chemokines (e.g. CXCL12) have been shown to participate in multiple skeletal cell regulation pathways and play a crucial role in the coordination of trafficking, differentiation, activity and apoptosis of skeletal cells under physiological and pathological circumstances (Gilchrist and Stern, 2015). In particular, based on studies *in vitro* (details in Chapters 2 and 3), it was found that CXCL12 achieves the most prominent induction among the chemokine family in apoptotic skeletal cells after Dex treatment, and that Dex-induced skeletal cell apoptosis and enhanced osteoclast differentiation could be molecularly linked by CXCL12 (Tang et al., 2016). However, *in vivo* studies are required for further verification.

Therefore, CXCL12 was selected as the candidate of interest in the current study, and it was hypothesized that Dex therapy-induced chondrocyte/osteocyte apoptosis triggers the induction of CXCL12 to promote chondroclast/osteoclast recruitment and formation, resulting in the uncontrolled cartilage/bone loss. Using a model of Dex treatment with or without CXCL12 blockade in young rats, the current study investigated Dex-induced structural, cellular and molecular damage/changes within the bone and marrow microenvironment and explored the potential protective effects of anti-CXCL12 neutralizing

antibody supplementation preventing Dex treatment-induced skeletal damage.

Materials and Methods

Animal trials and specimen collection

In brief, six-week-old male Sprague-Dawley rats were randomly divided into four groups: Dex/saline treatment + anti-CXCL12 neutralizing antibody/normal control IgG supplementation (Dex + IgG; Dex + anti-CXCL12; Saline + IgG; and saline + anti-CXCL12) groups (n=6/group). Rats in Dex treatment groups were orally gavaged with Dex (1 mg/kg) daily for 14 days in an effort to mimic the clinical regimen for acute lymphoblastic leukemia (ALL) maintenance treatment as described (Veerman et al., 2009); while rats in saline control groups received the same volume of saline gavage. In addition, half of the rats in Dex treatment/saline control groups additionally received intraperitoneal (I.P.) injection of anti-CXCL12 neutralizing antibody or normal control IgG, respectively as described (Kitaori et al., 2009; Kollmar et al., 2010). The injection of monoclonal mouse anti-CXCL12 neutralizing antibody (1 mg/kg; MAB310, R&D Systems Minneapolis, MN) commenced in the first day of Dex/saline treatment and repeated three times weekly for 2 weeks. Meanwhile, the corresponding isotype-matched IgG control antibody (mouse IgG1 isotype control; MAB002, R&D Systems) was injected with the same dose and schedule. By the end of day 14, rats in all groups were humanely sacrificed 24 hours after the final injection using carbon dioxide overdose. Left tibiae were dissected free of soft tissue, fixed in 10% formalin, decalcified in Immuocal solution (Decal Corporation, Tallman, NY) for 2 weeks at 4℃, then processed and embedded in paraffin for 4 μm thick sections for histological and immunohistochemical analyses as previously described (Xian et al., 2004b). The growth plates and metaphyses from the right tibiae were also collected and stored at -80℃ for gene expression analyses. The

animal procedures described above were approved by the Animal Ethics Committee of SA Pathology, South Australia.

Histomorphometric analyses of growth plate cartilage and metaphyseal bone

As a means to visualize the histology structures of the growth plate cartilage and bone, paraffin sections were dewaxed and stained by routine hematoxylin and eosin (H&E) as described (Xian et al., 2006). In addition, in order to determine the effects of Dex treatment with or without CXCL12 blockade on structural changes in growth plate cartilage, stained sections were subsequently used for morphometric measurements of growth plate thickness along the longitudinal axis by using an image analysis software (cellSens, Build14116; Olympus Corporation, Shinjuku, Tokyo, Japan) as described (Xian et al., 2006; Xian et al., 2004a). Histomorphometric analysis was also performed to measure the heights of primary spongiosa and bone volume fraction (bone volume/total tissue volume, BV/TV%) of second spongiosa in metaphysis as described (Xian et al., 2004a). Furthermore, tartrate-resistant acid phosphatase (TRAP) staining was conducted to evaluate the treatment effects on recruitment of cartilage resorbing chondroclasts and/or bone resorbing osteoclasts as previously described (Fan et al., 2009).

Hoechst staining and ISNT reaction for apoptosis labeling

To determine the effects of Dex treatment on chondrocyte apoptosis, cell nuclear morphology staining using fluorescent Hoechst dye (Molecular Probes, Eugene, OR) was carried out as described (Xian et al., 2004a), analyses under a fluorescent microscope. The cells with characteristic condensed or fragmented nuclei morphology were identified as apoptotic cells and the density of apoptotic chondrocytes (cells/mm^2) was calculated as described (Xian et al., 2004a). In addition, *in situ* DNA nick translation (ISNT) reaction by using a nick

translation mixture (Roche, North Ryder, NSW, Australia) was conducted as described (Fan et al., 2012; Noble and Stevens, 2003) for the identification and quantitation of osteocyte apoptosis in response to Dex treatment. ISNT-positive apoptotic osteocytes within trabecular bone were quantified and the apoptotic osteocytes density (cells/mm^2) was then calculated as described (Fan et al., 2012).

Immunohistochemical analyses

Immunohistochemistry was performed as described (Georgiou et al., 2012b) to detect the presence of CXCL12 and potential changes of its protein expression within the growth plate and metaphysis of rats with/without Dex treatment ± CXCL12 blockade. In brief, 4 μm paraffin sections were dewaxed, quenched with 3% hydrogen peroxide in 50% methanol, treated with an antigen retrieval solution (pH 9.0; Dako, North Sydney, NSW, Australia) at 80 ^{0}C for 1.5 h, and blocked with 10% normal rabbit serum. The sections were then incubated with monoclonal anti-CXCL12 antibody (25 μg/ml; MAB310; R&D Systems) at 4 ^{0}C overnight. After washing, the sections were incubated with the rabbit anti-mouse biotinylated secondary antibody (1:400; Dako), followed by the incubation with horseradish peroxidase solution (Merck KGaA, Darmstadt, HE, Germany). The immunological reaction was visualized by 3, 3'-diaminobenzidine (DAB) substrate (Dako) and hematoxylin was subsequently employed for counterstaining.

Real-time qRT-PCR

As a means to investigate the treatment effects on expression of regulatory genes in bone and related to osteoblast, osteoclast and adipocyte formation/function, real-time quantitative RT-PCR was conducted with RNA obtained from frozen metaphyseal specimens as described (Fan et al., 2012). Briefly, total RNA was isolated by using TRI reagent (Sigma) and single

stranded cDNA was then synthesized using iScript™ Reverse Transcription Supermix for RT-Qpcr kit (Bio-Rad, Hercules, CA). Primers for the genes of interest (Table.4-1) were designed using NCBI Primer-Blast software and supplied by GeneWork (Adelaide, SA, Australia). SYBR Green PCR assays (Bio-Rad) were carried out in triplicate on cDNA samples in a CFX PCR System (Bio-Rad) and relative quantification was calculated with using the $2^{-\Delta}$CT method relative to the endogenous control gene Cyclophilin-A (CycA).

Table 4-1. PCR primer sequences used for RT-PCR gene expression analyses.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Runx2	TCACAAATCCTCCCCAAGTGG	GAATGCGCCCTTAAATCACTGA
OSX	GCTTTTCTGTGGCAAGAGGTTC	CTGATGTTTGCTCAAGTGGTCG
OCN	GCTGGCCCTGACTGCATTCTG	ATTCACCACCTTACTGCCCTCCTG
SPRF-1	CCCGAGATGCTCAAATGTGAC	AGATGTTCGATGATGGCCTCC
SOST	CAACCAGACCATGAACCGGG	AAGCGGGTGTAGTGCAGCTC
Dkk-1	GGTTCTTGGTCGTGCTTTCA	CTTGATCGCGTTGGAATTGA
RANKL	CCGTGCAAAGGGAATTACAAC	ATATGCCGTTGCACACTGCTT
OPG	CACAGCTCGCAAGAGCAAACT	AAGCTTGACACGCACCAGTCT
C/EBPα	TCGCCATGCCGGGAGAACTCTAAC	CTGGAGGTGGCTGCTCATCGGGG
CXCL12	ATCAGTGACGGTAAGCCAGTCA	TGCACACTTGTCTGTTGTTGCT
CycA	CGTTGGATGGCAAGCATGTG	TGCTGGTCTTGCCATTCCTG

Ex vivo mineralization assay

An *ex vivo* mineralization assay was performed to examine the effects of Dex treatment ± CXCL12 blockade on osteogenic potential of bone marrow stromal cells as described (Fan et al., 2016). In brief, bone marrow was flushed out with basal medium containing alpha minimum essential medium (α-MEM; Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 100 U/ml penicillin-100 μg/ml streptomycin

(Invitrogen), and 15 mM Hepes buffer (PH7.4; Sigma). Bone marrow mononuclear cells were then isolated by using lymphoprep solution (Axis-Shield PoC AS, Oslo, Norway). Following an overnight culture, the non-adherent hematopoietic cells were collected for osteoclast formation assay, while plastic adherent bone marrow stromal cells were used for mineralization assay. These bone marrow stromal cells were cultured in 6-well plates at a density of 4×10^6 cells/well in basal medium for 7 days, followed by cultured with basal medium supplemented 10mM β-glycerol-2-phosphate (Sigma) and 10 nM Dex (Sigma) for additional 21 days. The mineralization behavior of bone marrow stromal cells was detected by Alizarin red S (AR-S) staining and the absorbance was measured at a wavelength of 405 nm to determine the intensity of AR-S stains as previously described (Gregory et al., 2004).

Ex vivo osteoclast formation assay

To assess the treatment effects on osteoclast formation in the bone marrow, an *ex vivo* osteoclast formation assay was carried out as described (Fan et al., 2016). Briefly, the collected non-adherent hematopoietic cells (see above) were reseeded at a density of $1x10^6$ cells/well in basal medium supplemented with 10 ng/ml macrophage colony-stimulating factor (M-CSF; Peprotech, Rocky Hill, NJ) for one day. The cells were subsequently cultured in basal medium supplemented with 10 ng/ml M-CSF and 30 ng/ml receptor activator of nuclear factor kappa-κB ligand (RANKL; Peprotech) for seven days with media refreshed twice prior to fixation in 10% formalin, followed by TRAP staining. TRAP positive ($TRAP^+$) cells with multinucleated ($\geqslant$3 nuclei) were counted as osteoclasts and osteoclast formation was expressed as the density of multinucleated osteoclasts (number/mm^2 culture area) using image analysis software (cellSens; Olympus Corporation).

Statistical Analyses

Data were represented as mean ± SEM and analysed by one-way analysis of variance (ANOVA) and Tukey's post-hoc by using Prims 6 software (GraphPad, San Diego, CA), where a P-value < 0.05 is accepted to achieve statistically significance. In the figures, the symbols *, ** and *** represented $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Results

Treatment effects on chondrocyte apoptosis, chondroclast density, and CXCL12 induction in growth plate

In the current study, chondrocyte apoptosis identified by Hoechst dye and hematoxylin staining was found to be expressively induced within the growth plate after Dex treatment, particularly at the bottom of chondrocyte columns in hypertrophic zone (Fig.4-1A). Density quantification of apoptotic chondrocytes showed a statistically significant increase in Dex-treated groups compared to saline-treated groups ($P<0.01$) (Fig.4-1B). In addition, without the supplementation of anti-CXCL12 neutralizing antibody, the density of TRAP+ cartilage-resorbing chondroclasts was observed to be significantly increased after Dex treatment ($P<0.05$) (Fig.4-1C, E). However, this alteration was restored to the untreated level following anti-CXCL12 neutralizing antibody supplementation ($P<0.05$).

Furthermore, to investigate the treatments on CXCL12 induction in growth plate, qRT-PCR analysis showed that the expression levels of CXCL12 were significantly upregulated following Dex treatment in comparison to saline controls ($P<0.01$), which is preserved by the supplementation of anti-CXCL12 neutralizing antibody ($P<0.05$) (Fig.4-1F). Furthermore, immunohistochemical localization of CXCL12 was carried out. CXCL12 positivity was not

or only weakly detected in the growth plate from saline-treated rats. However, positive staining for CXCL12 was extensively found at the bottom of chondrocyte columns within the hypertrophic zone, particularly close to or along the chondro-osseous transitional zone from the rats with Dex + control IgG combination treatment. Interestingly, the obvious reduction in CXCL12-staining was observed within the same regions from the Dex treated-rats supplemented with the anti-CXCL12 neutralizing antibody (Fig.4-1D).

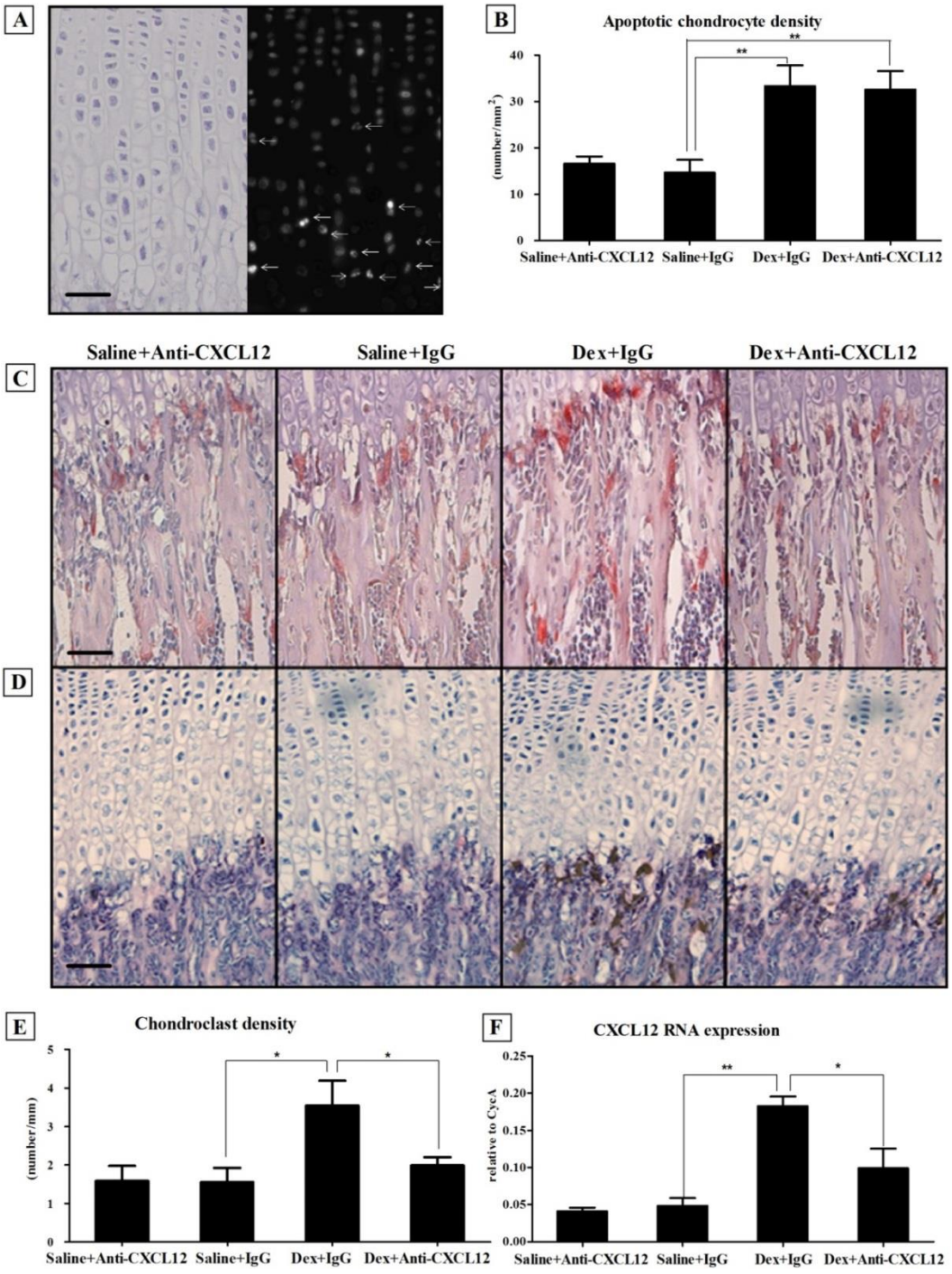


Figure 4-1. The effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on chondrocyte apoptosis, chondroclast recruitment, and CXCL12 induction in tibia of young rats. (A) Representative images of apoptotic chondrocytes (solid arrows) assessed by Hoechst dye and haematoxylin staining within growth plate from young rats between Dex + normal control IgG/anti-CXCL12 neutralizing antibody treatment groups. **(B)** The densities of apoptotic chondrocytes in the growth plate in four treatment groups. **(C)** Representative histology images of chondroclasts (red colour) identified by TRAP staining within metaphyseaisl chondro-osseous transitional zone of proximal tibia in four treatment groups. **(D)** Representative images of immunohistochemical localization of CXCL12 (brown colour) in metaphyseaisl chondro-osseous transitional zone. **(E)** The densities of chondroclasts within chondro-osseous transitional zone in four treatment groups. **(F)** The treatment effects on mRNA expression of CXCL12 (relative to Cyclophilin-A or CycA) in growth plate. Scale bar on panels A, C, and D = 50 μm, 100 μm and 100 μm, respectively.

Treatment effects on structural changes in growth plate and adjacent metaphyseal primary spongiosa

To determine the treatment effects on structural changes in growth plate and adjacent metaphyseal primary spongiosa in tibia, growth plate thickness and metaphyseal primary spongiosa height were analyzed for the rats of the four treatment groups. Within saline-treated rats, no structural changes were observed between anti-CXCL12 neutralizing antibody and control IgG supplementation groups (Fig.4-2). However, histomorphometric measurements revealed that the Dex treatment in the absence of anti-CXCL12 neutralizing antibody supplementation caused a significant reduction in both growth plate thickness and primary spongiosa height when compared to the saline + control IgG treatment ($P<0.01$, $P<0.05$ and $P<0.05$, respectively), which was restored by the anti-CXCL12 neutralizing antibody supplementary treatment (Fig.4-2).

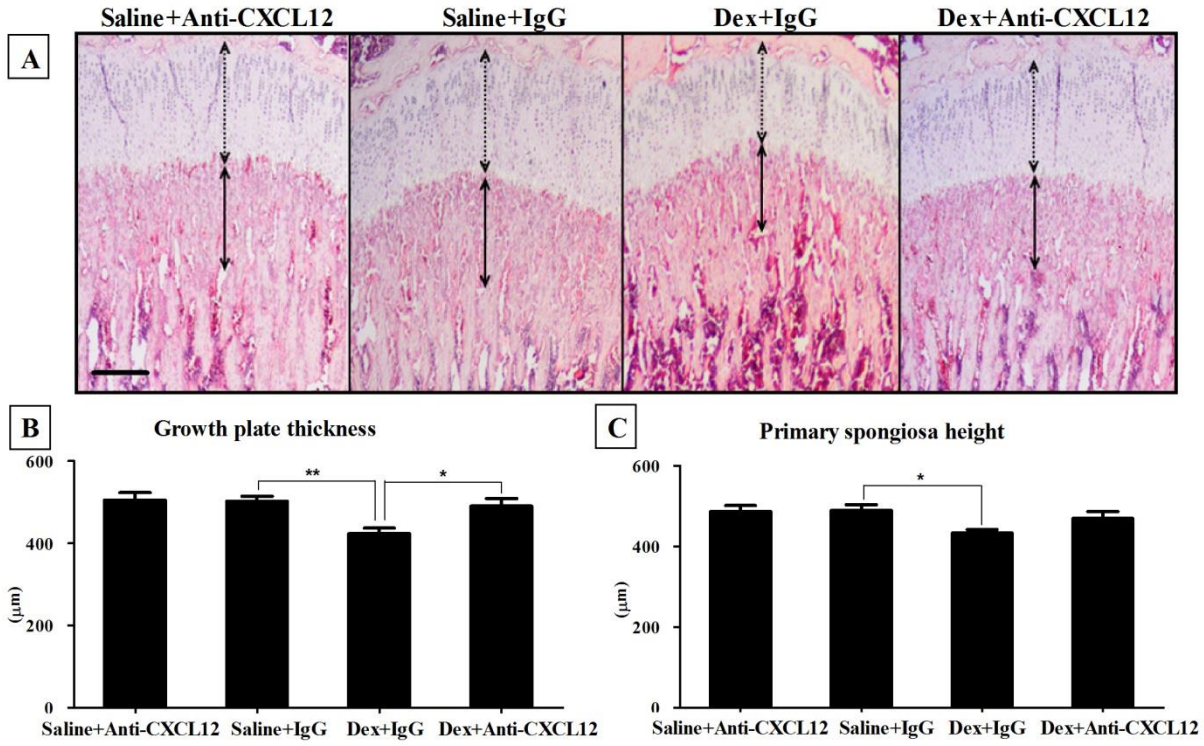


Figure 4-2. The effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on growth plate and primary spongiosa heights in tibia of young rats. Representative images of haematoxylin and eosin stained sections showing effects of four different treatments on **(A)** growth plate thickness (dashed lines) and primary spongiosa heights (solid lines). Growth plate thickness and primary spongiosa height were measured and illustrated in **(B-C)**, respectively. Scale bar on panel A = 400 μm.

Treatment effects on the trabecular bone volume and structure at the metaphysis secondary spongiosa

Consistent with the observed structural changes in growth plate and primary spongiosa, histomorphometric measurements found a considerably decreased bone volume fraction (trabecular bone volume/total tissue volume, BV/TV %) within the secondary spongiosa after Dex treatment ($P<0.01$), in which trabecular density and thickness were significantly reduced ($P<0.05$ and $P<0.001$, respectively) and trabecular spacing was correspondingly increased ($P<0.01$). Interestingly, these alterations were observed to be noticeably restored by anti-CXCL12 neutralizing antibody supplementation (Fig.4-3).

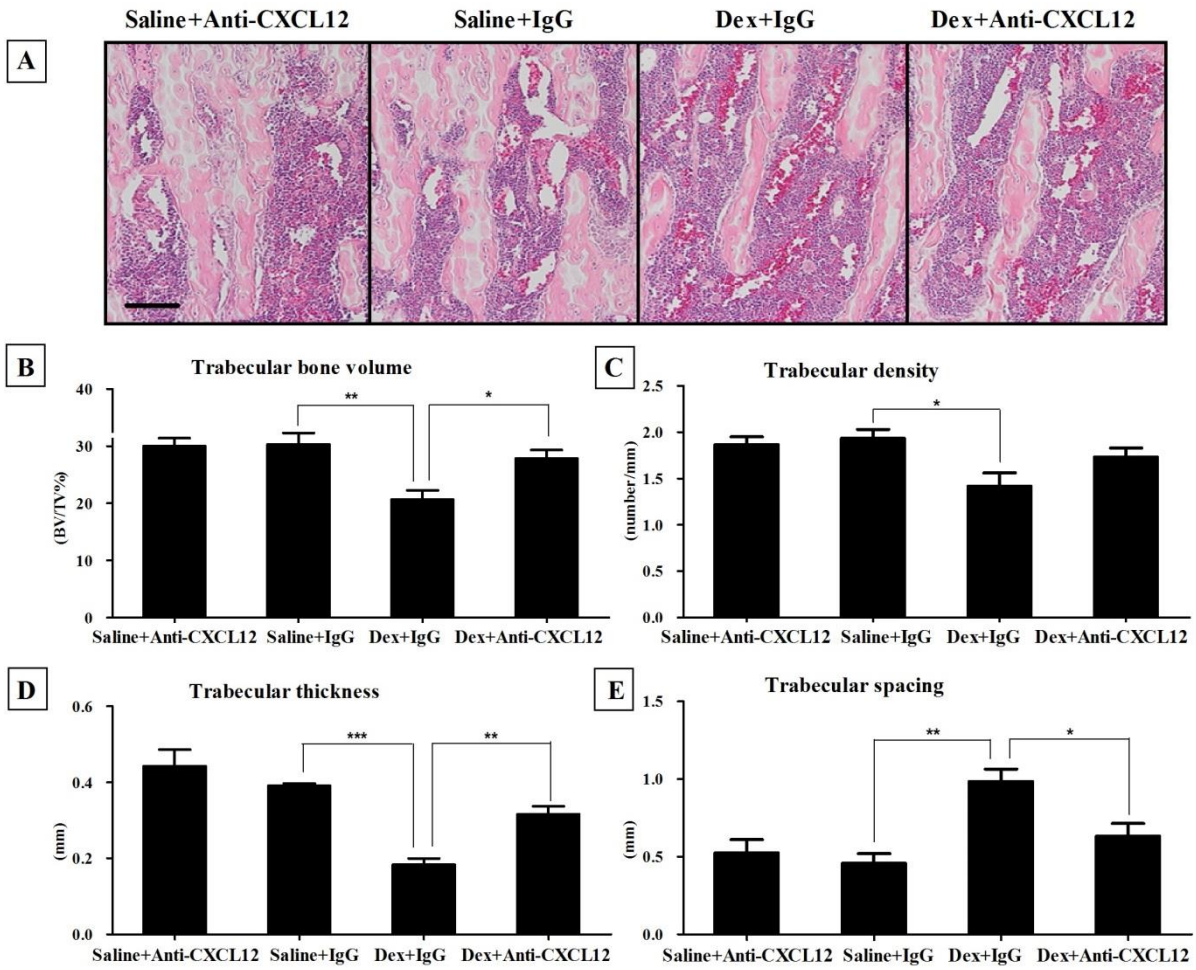


Figure 4-3. The effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on trabecular bone volume and structure within metaphysis secondary spongiosa in tibia of young rats. Representative images of haematoxylin and eosin stained sections showing effects of four different treatments on **(A)** the trabecular bone volume at the metaphysis secondary spongiosa. Trabecular bone volume fraction BV/TV, density, thickness and spacing were illustrated in **(B-E**), respectively. Scale bar on panel A = 150 μm.

Treatment effects on osteoblast density and osteogenic differentiation potential

Although a trend towards a reduction in osteoblast density was observed in Dex + control IgG treatment group, no statistically significant differences were noted among four treatment groups (Fig.4-4A). Consistent with these findings, no significant treatment effects were observed in mRNA expression levels of osteoblast-specific bone protein osteocalcin (OCN) and transcription factor osterix (Osx) (Fig.4-4B, D). However, the expression levels of early osteogenic transcription marker Runt-related transcription factor 2 (Runx-2) were obviously

downregulated ($P<0.05$) following Dex treatment when compared to saline treatment groups (Fig.4-4C). In addition, as shown in Figure 4-4E, mineralized matrix nodules and calcium deposition (stained bright orange-red color by using AR-S) were prominently observed among the saline control groups but less so in Dex-treated groups in an *ex vivo* mineralization assay. Consistently, quantification of AR-S showed a significant decrease in Dex-treated groups (both $P<0.001$) (Fig.4-4F). Furthermore, RT-PCR analysis was also carried out for the expression of major antagonists of Wnt signaling such as secreted frizzled related protein-1 (SFRP-1), dickkopf-1 (Dkk-1) and sclerostin (encoded by SOST gene), which are known to suppress Wnt signaling and therefore osteogenic differentiation. While Dex treatment appeared to cause slight effects on SFRP-1 expression (Fig.4-4G), the expression levels of Dkk-1 and SOST were markedly enhanced by Dex treatment ($P<0.05$ and <0.01, respectively) (Fig.4-4H, I).

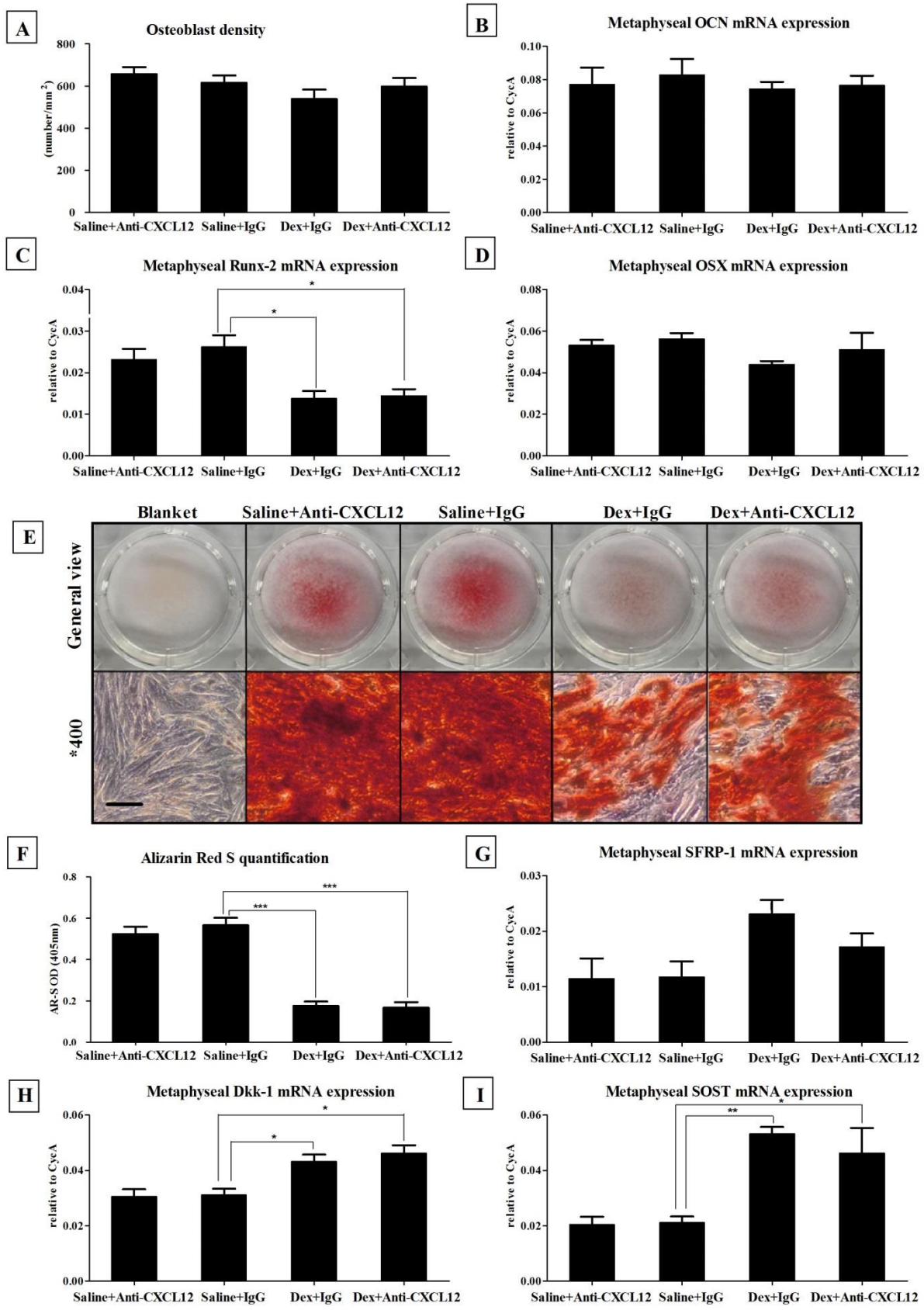


Figure 4-4. The effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on osteoblast density, expression of osteogenesis-related genes, and bone marrow stromal cell osteogenic differentiation potential in tibia of

young rats. The densities of osteoblasts along trabecular bone surface within metaphysis bone in four treatment groups were measured in **(A)**. The treatment effects on mRNA expression of osteogenesis-related genes (relative to Cyclophilin-A or CycA): **(B)** Osteocalcin (OCN), **(C)** Osterix (Osx) and **(D)** Runt-related transcription factor 2 (Runx-2). Osteoblastogenic differentiation potential of bone marrow stromal cells was determined by Alizarin Red S (AR-S) staining in an *ex vivo* mineralization assay. **(E)** Representative images of stained plates (upper) and microscopic view at a magnification 400 times (lower). AR-S staining extraction was measured by the absorbance at 405 nm (F). The treatment effects on mRNA expression of Wnt antagonists: **(G)** secreted frizzled related protein-1 (SFRP-1), **(H)** dickkopf-1 (Dkk-1) and **(I)** sclerostin (encoded by SOST). Scale bar on panel E = 50 μm.

Treatment effects on osteocyte apoptosis and CXCL12 induction within metaphyseal zone

ISNT labeling was conducted to examine osteocyte apoptosis in response to Dex treatment (Fig.4-5A). It was observed that Dex treatment resulted in a significant increase in the density of apoptotic osteocytes in bony trabeculae within metaphysis region with/without the supplementation of anti-CXCL12 neutralizing antibody ($P<0.001$) (Fig.4-5B). Although no statically significant differences in CXCL12 mRNA expression were noted among four treatment groups, an observable but insignificant trend of increase was found in Dex + control IgG combination treatment group (Fig.4-5C). In addition, immunohistochemistry analysis showed that the alterations in the population of $CXCL12^+$ osteocytes within lacunae upon various treatments are similar as those in apoptotic osteocytes. Briefly, no $CXCL12^+$ cells were detected within metaphyseal zone of tibia from saline-treated rats, while positive staining for CXCL12 was extensively observed in osteocytes within secondary spongiosa trabeculae in metaphysis from Dex-treated rats (Fig.4-5D).

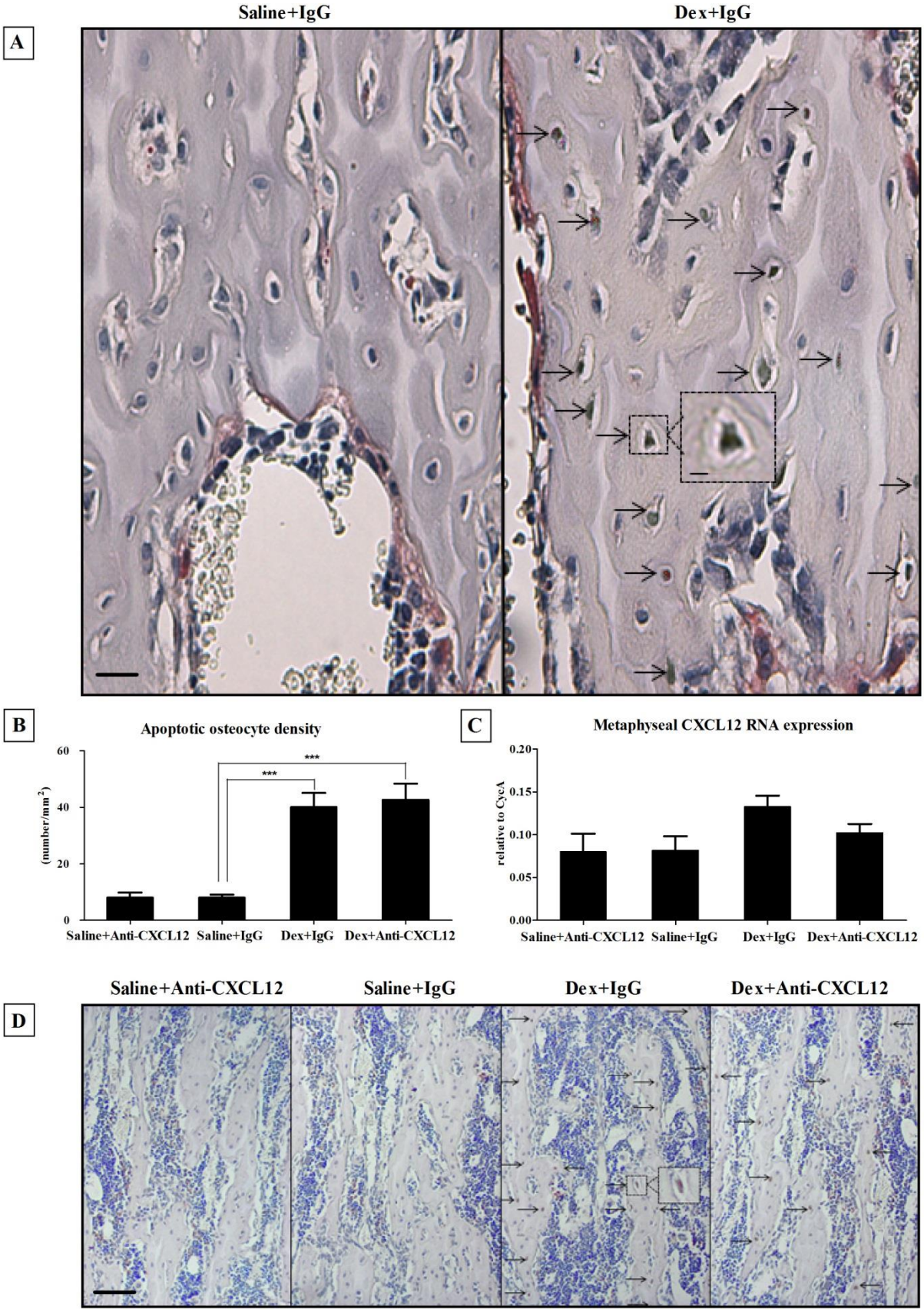


Figure 4-5. The effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on osteocyte apoptosis, and CXCL12 induction in tibia of young rats. (A) Representative histology images of apoptotic osteocytes (solid arrows)

detected by *In Situ* Nick Translation (ISNT) labeling within metaphysis from the young rats between Dex + normal control IgG/anti-CXCL12 neutralizing antibody treatment groups. **(B, C)** The densities of apoptotic osteocytes and mRNA expression levels of CXCL12 within metaphysis bone in four treatment groups. **(D)** Representative images of immunohistochemical localization of CXCL12 in secondary spongiosa trabeculae. Arrows point to labeled osteocytes. Scale bar on panels A = 50 μm and D = 200 μm.

Treatment effects on osteoclast density and osteoclastogenic differentiation potential

Density measurements for bone-resorbing osteoclasts showed similar patterns of changes upon various treatments as for chondroclasts (Fig.4-6A). It was found that more osteoclasts were obviously recruited following Dex treatment ($P<0.001$), which was strikingly inhibited by the supplementation of anti-CXCL12 neutralizing antibody ($P<0.001$) (Fig.4-6B). In addition, mRNA expression analysis showed that the RANKL / osteoprotegerin (OPG) ratio, which is the determinant for osteoclastogenesis (Weinstein, 2012), was notably upregulated in Dex + control IgG treatment group in comparison to saline controls ($P<0.05$), while anti-CXCL12 neutralizing antibody appeared to preserve their expression levels after Dex treatment (Fig.4-6C).

Furthermore, the impact of treatments on osteoclastogenic differentiation potential of the bone marrow cells was examined by an *ex vivo* osteoclast formation assay. Although osteoclasts or osteoclast-like cells were observed in all the cultures from four treatment groups (Fig.4-6D), a significantly increased population of osteoclasts was found in Dex + control IgG treatment group ($P<0.001$). Consistently, supplementation with anti-CXCL12 neutralizing antibody was shown to considerably suppress Dex-improved osteoclastogenesis potential ($P<0.001$) (Fig.4-6E).

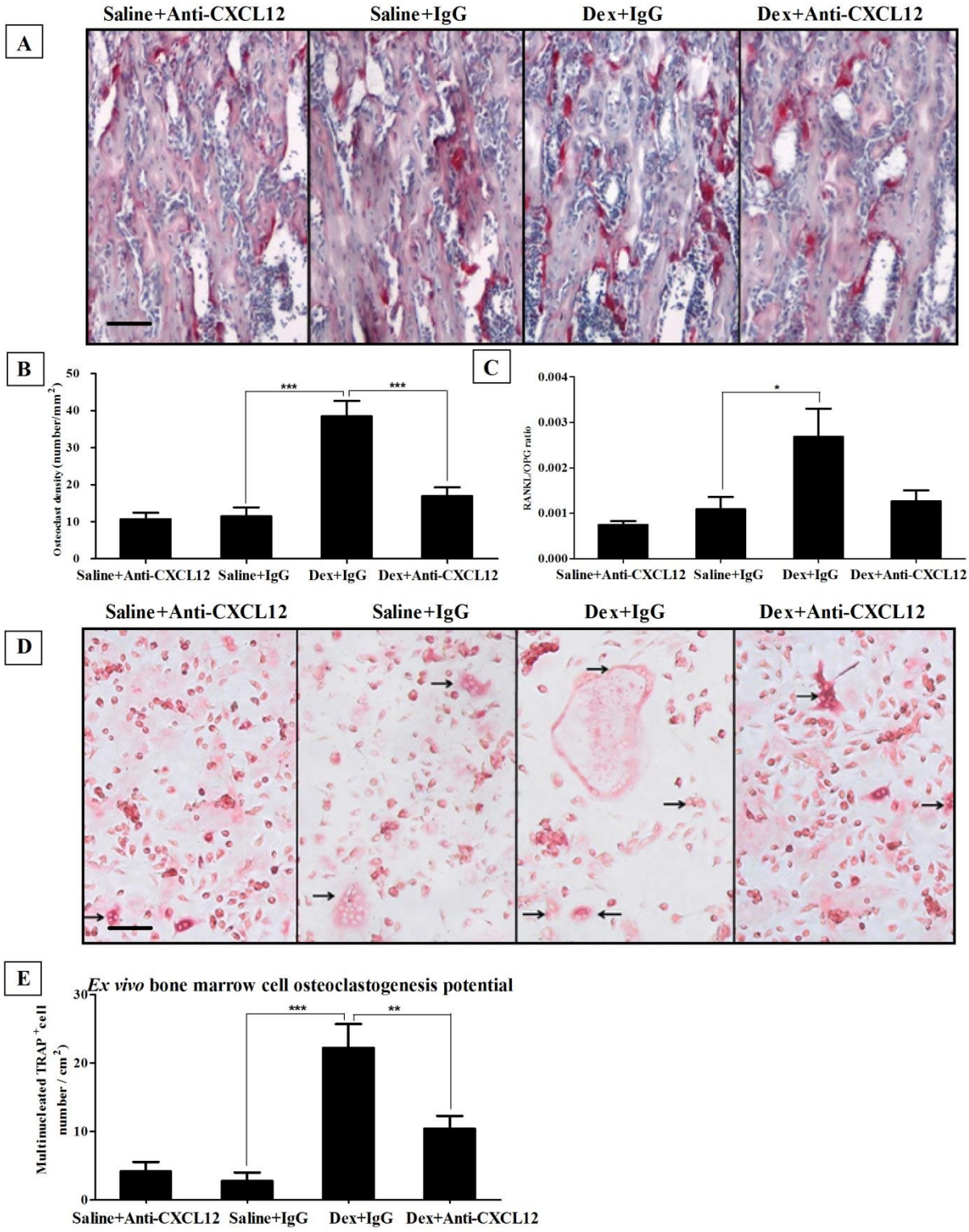


Figure 4-6. Effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on osteoclast density, expression of osteoclastogenesis-related genes and bone marrow cell osteoclastogenic differentiation in tibia of young rats. (A) Representative histology images of TRAP positive osteoclasts (red color) along trabecular bone surface at the secondary spongiosa. **(B, C)** Osteoclast density within the secondary spongiosa and mRNA expression ratio of key osteoclastogenic factor RANKL and inhibitor OPG. **(D)** Representative images of multinuclear TRAP positive osteoclasts (solid arrows) in *ex vivo* osteoclast formation assay. **(E)** Osteoclast density in *ex vivo* osteoclast formation

assay. Scale bar on panel A = 100 μm (also applies to D).

Treatment effects on adipocyte density and adipogenic differentiation potential

To investigate the treatment effects on adipogenesis in the bone marrow microenvironment, the density of adipocyte was quantified at the lower secondary spongiosa. It was revealed that Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation caused a significant increase in adipocyte density ($P<0.001$) (Fig.4-7A, B). In addition, RT-PCR analysis showed that mRNA expression levels of adipogenesis regulatory gene CCAAT/enhancer binding protein alpha (C/EBPα) were in a similar trend of changes upon various treatments (Fig.4-7C).

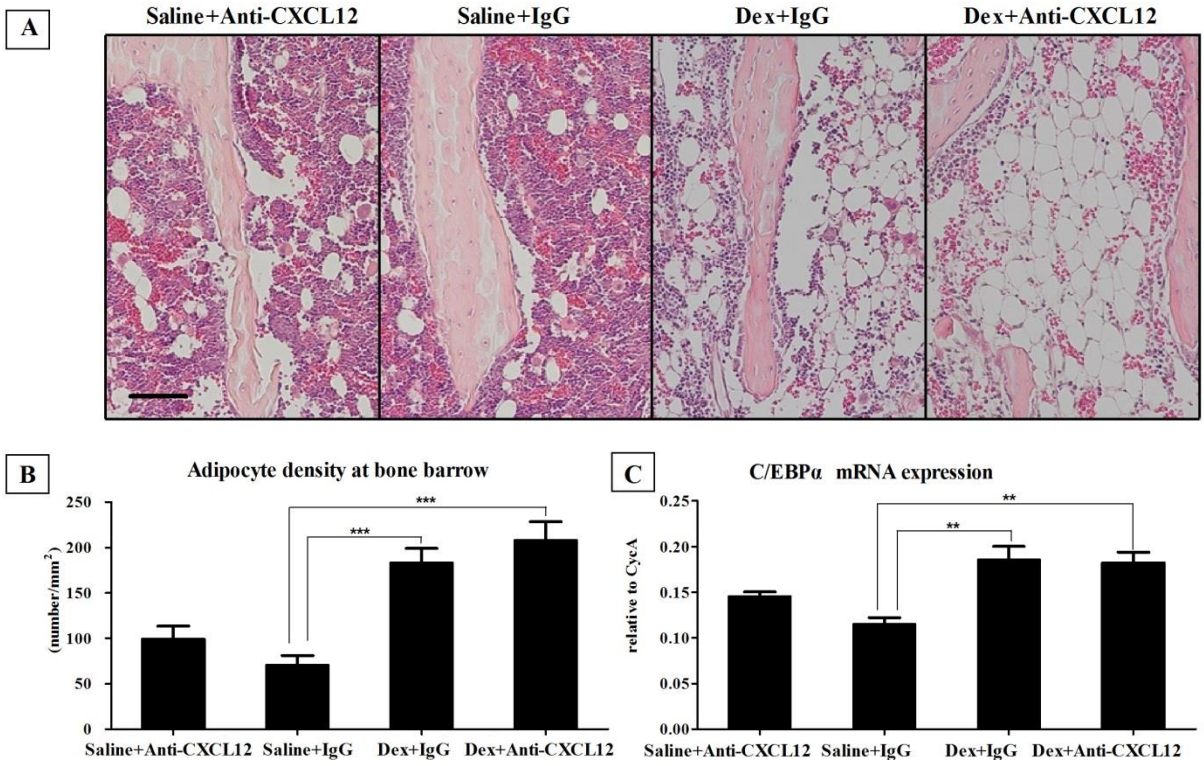


Figure 4-7. Effects of Dex treatment with or without anti-CXCL12 neutralizing antibody supplementation on bone marrow adiposity and expression of adipogenesis-related genes. (A) Representative histology images of lower metaphyseal secondary spongiosa in four treatment groups. **(B)** Adipocyte cell density in the bone marrow. **(C)** mRNA expression of adipogenesis-related gene CCAAT/enhancer binding protein alpha (C/EBPα) (relative to Cyclophilin-A or CycA). Scale bar on panel A = 200 μm.

Discussion

Dex is a synthetic glucocorticoid compound and has been widely used in clinical practice. However, intensive use of Dex treatment has been shown to cause severe adverse effects on the skeleton, for which the underlying mechanism still remains largely unknown and there is a lack of an effective treatment approach. Based on our recent studies (details in Chapters 2 and 3), it was hypothesised that chondrocyte/osteocyte apoptosis following Dex treatment could trigger the expression of chemokine CXCL12, which in turn stimulates chondroclast/osteoclast recruitment and formation, resulting in the cartilage/bone loss. Therefore, in the current study, a rat Dex treatment + CXCL12 blockade model was employed to further investigate the potential involvement of CXCL12 in Dex-induced bone growth defects. A significant reduction in growth plate thickness, primary spongiosa height and trabecular bone volume were observed after Dex treatment, which were partially owing to induced chondrocyte/ osteocyte apoptosis and enhanced chondroclast/osteoclast recruitment and activity. These Dex-induced alterations in skeletal cell events were shown to be potentially linked by CXCL12, as CXCL12 was found induced at the apoptotic chondrocytes and osteocytes and blocking CXCL12 function using an anti-CXCL12 neutralizing antibody was found to have the potential to prevent Dex-induced cartilage/bone loss.

Dex treatment induced the dysfunction of growth plate

Previous studies have shown that intensive Dex therapy causes severe growth plate dysfunction and the consequent bone growth defects (Zaman, 2010). Although the underlying molecular mechanisms remain largely unknown, it has been suggested that Dex exerts direct effects on the differentiation, activity, and apoptosis of chondrocytes, resulting in the dysfunction of growth plate (Mushtaq et al., 2004). Chondrocytes in the growth plate, which

arrange in columns and proceed through distinct stages of maturation in these organized columns (Ballock and O'Keefe, 2003), are known to perform highly specialized synthesis and secretion of cartilage matrix, which acts as a scaffold for longitudinal bone growth (Xian, 2007). Apoptosis is the default fate for chondrocytes and principally identified at the metaphyseal chondro-osseous transitional zone which is adjacent to growth plate hypertrophic zone (Green, 1998). In particular, chondrocyte apoptosis has been demonstrated to play a crucial role in the shaping of long bones during development (Mackie et al., 2011). However, aside from the inhibition of chondrocyte proliferation, the adverse effects of Dex therapy on the skeleton are evident to be partly induced by an increase in chondrocyte apoptosis, principally in terminally differentiated hypertrophic chondrocytes (Chrysis et al., 2003), which is associated with thinning of the growth plate and bone growth impairment (Zaman et al., 2012). In addition, osteoclasts identified within chondro-osseous transitional zone are characterized as chondroclasts which are specifically involved in the resorption of calcified cartilage (Vu et al., 1998). Approximately 70% cartilage matrix is degraded by chondroclasts, whilst the cartilaginous remnants serves as a template for bone matrix deposition by osteoblasts to form trabecular bones (Ballock and O'Keefe, 2003). Previous studies have shown that the inhibition of chondroclast activities results in a reduction of calcified cartilage resorption, leading to an increase of bone volume (Turner et al., 1994). In the current study, Dex-induced chondrocyte apoptosis was expressively detected mainly within the hypertrophic zone of growth plate, and the density of apoptotic chondrocytes was increased from $14.6 \pm 3.1/mm^2$ in saline + control IgG group to $33.3 \pm 4.3/mm^2$ in Dex + control IgG group (increased by 128.1%). In addition, significantly augmented recruitment of resorbing chondroclasts was also observed following Dex treatment (chondroclast density increased by 128.4%), which may contribute to interference of calcified cartilage feeding into the adjacent metaphysis for subsequent bone deposition. Thus, collectively, Dex treatment

considerably reduced growth plate thickness (by 20%), which is at least in part attributed to enhanced chondrocyte apoptosis and osteoclast recruitment, and therefore caused the deficiency of calcified cartilage scaffold for subsequent bone formation, resulting in the suppression of longitudinal growth and bone mass accumulation.

Dex treatment induced the disruption of bone homeostasis

During normal endochondral ossification, newly formed primary spongiosa, which is composed of the calcified cartilage delivered from the adjacent growth plate, is further modeled and/or remodeled to the secondary spongiosa, which is made up with enlarged mineralized trabecular bones, by osteoblasts and osteoclasts (Xian, 2007). Under physiological circumstances, there is a balance between the coupled bone formation and resorption, which is responsible for the constant but subtle reshaping of bones (Rosen et al., 2009). However, in situations such as glucocorticoid therapy, this ongoing balance is disrupted, leading to the impaired endochondral ossification and bone remodelling (Hardy and Cooper, 2009). Consistent with the decreased growth plate thickness, sharp reductions in primary spongiosa height (decreased by 15.8%) and trabecular bone volume within secondary spongiosa (decreased by 32.1%) were observed in Dex + control IgG -treated rats in comparison to saline controls, suggesting that significant bone loss was induced after Dex treatment at 1 mg/kg/day for 14 consecutive days. In particular, in comparison to the reduced trabecular density (decreased to 73.3%), Dex treatment appeared to have more significant effects on trabecular thickness (decreased to 46.9%) and the corresponding trabecular spacing (increased to 215.5%), indicating more excessive bone resorption than inhibited bone formation in the current *in vivo* model.

Intense use of Dex treatment has been illustrated to stimulate osteoclast differentiation,

which is related to the upregulation of RANKL (Hofbauer and Rauner, 2009) and the inhibition of OPG (Sasaki et al., 2002). The increased RANKL/OPG ratio has been confirmed to directly result in the induced osteoclast precursor recruitment, which in turn translates into enhanced osteoclast formation and prolonged osteoclast lifespan and leads to promoted bone resorption (Jia et al., 2006). However, the mechanisms by which Dex induces osteoclast recruitment and differentiation still remain largely unknown. In the current study, histomorphometric analyses showed that the density of osteoclasts in the metaphyseal bone was strikingly increased to 335.3% in response to Dex treatment. Meanwhile, consistent with the elevated osteoclast density on the bone surface, *ex vivo* osteoclastogenesis studies showed the obviously augmented formation (increased by 599.2% compared to saline control) of $TRAP^+$ multinucleated osteoclasts from the bone marrow cells of rats following Dex treatment in control IgG-supplemented rats, suggesting Dex treatment significantly improved osteoclastogenic differentiation potential for the bone marrow cells. Consistently, although RT-PCR gene expression analyses indicated that the expression levels of RANKL and OPG in the metaphysis have a similarly elevated trend after Dex treatment (data not shown), the RANKL/OPG ratio is considerably increased to 248.6%. In accordance with these findings, enhanced osteoclast formation and activity are significantly induced after Dex treatment, contributing towards the excessive bone resorption.

On the other hand, although it has been demonstrated that Dex treatment could cause a negative effect on osteoblast formation (Bouvard et al., 2010), the current study found no statistically significant differences in osteoblast densities along trabecular bone surface between saline and Dex treatment groups, suggesting Dex treatment had insignificant or slight effects on osteoblast activities under circumstances, which is inconsistent with the observed alterations in trabecular bone structure and volume following Dex treatment. Gene

expression studies on specific osteoblastogenesis-related molecule and transcription factors such as runt-related transcription factor 2 (Runx2), Osterix (Osx) and osteocalcin (OCN) in the metaphysis were therefore carried out to further investigate the treatment effects of Dex on bone formation. While Runx2 is regarded as a molecular marker for the early stage of osteoblastogenic differentiation, Osx acts downstream of Runx2 and also exerts considerable influence on osteoblastogenesis, which is specifically critical to the commitment of preosteoblastic cell differentiation into mature osteoblasts (Kobayashi and Kronenberg, 2005), and thus is considered as one of the molecular markers for the late stage of osteoblastogenic differentiation. In addition, OCN is the most abundant non-collagenous protein of bone matrix and evident to exert the promoting effects on bone formation and mineralization (Zoch et al., 2016). In the current study, consistent with the change pattern of the osteoblast density, an observable but insignificant decrease in expression of metaphyseal Osx and OCN was observed upon Dex treatment. However, the mRNA expression levels of Runx2 were markedly decreased to 189.8% after Dex treatment, indicating the process of osteoblast differentiation could be repressed by Dex. Consistently, in comparison to Dex-treated groups, conspicuously observed mineralized matrix nodules and higher-intensity of Alizarin-Red staining were noted in bone marrow stromal cell osteogenic cultures from the saline control groups, which suggest osteoblastogenesis and mineralization potential is markedly inhibited in the bone marrow after Dex treatment in the current study.

Furthermore, it is well-established that canonical Wnt/β-catenin signalling is an enticing pathway implicated in bone development, particularly important in promoting osteoblast differentiation but suppressing adipogenic differentiation in the bone marrow (Hardy and Cooper, 2009; Macsai et al., 2008). Previous studies have also illustrated that Wnt/β-catenin signaling pathway could be attenuated by Dex administration through up-regulated

expression of Wnt antagonists such as Dkk-1 and SFRP-1 as well as down-regulated expression of Wnt receptor complex components Fz-4 and Fz-7 and downstream transcription factor LEF-1, which result in the suppression of β-catenin signaling, leading to the ultimate inhibition of osteoblast differentiation (Bouvard et al., 2010; Georgiou et al., 2012a). Despite the slight increasing trend in SPRF-1 expression, the levels of Dkk-1 and SOST mRNA expression in the metaphysis were found to be strikingly upregulated to 138.7% and 252.4%, respectively, after Dex treatment. Taken together, these findings showed that Dex treatment exert an inhibitory effect on osteoblast differentiation which may be associated with an increase in Wnt antagonist expression and a potential defect Wnt/β-catenin signaling.

Aside from the inhibition of osteoblast formation and function, Dex treatment has been illustrated to be responsible for the induction of osteocyte apoptosis (O'Brien et al., 2004). As the terminally differentiated osteoblast lineage cells, only approximately 2.5% osteocytes undergo apoptosis each year under physiologic conditions (Manolagas and Parfitt, 2010). The normal rate of osteocyte apoptosis is essential for skeletal health (Jilka et al., 2014). However, Dex-induced pro-apoptotic effects on osteocytes could potentially disrupt the lacunar-canalicular network and lead to the defeat of signal detecting, resulting in an inhibition of damaged bone replacement or local remodeling (Jilka et al., 2013). Consistently, an obvious increase in densities of apoptotic osteocytes (increased by 403.3%) within bony trabeculae in metaphysis was observed in Dex + control IgG -treated rats in comparison to saline controls. Collectively, the current study observed that damaging effects on bone homeostasis induced by Dex treatment are mainly owing to the significantly stimulated osteoclast formation and osteocyte apoptosis.

Dex treatment induced the alteration in bone marrow adipocyte population

Bone marrow MSCs are pluripotent progenitor cells which can differentiate into various lineages, including osteogenic and adipogenic lineages (Pittenger et al., 1999), suggesting there exists a reciprocal switch in bone versus fat volume within the bone marrow duo to the common precursor shared by osteoblasts and adipocytes (Bonfield and Caplan, 2010). Previous studies have shown that intensive Dex treatment can cause a significant switch in MSC commitment from osteogenesis to adipogenesis (Canalis et al., 2007), through the inhibition of osteogenic transcription factor Runx2 as well as the induction of C/EBPα which in turn transcriptionally activates chief adipogenic regulator peroxisome proliferator-activated receptor gamma (PPARγ2) to promote adipogenesis (Shi et al., 2003). In the current study, aside from the downregulated expression levels of Runx2, the markedly upregulated expression levels of C/EBPα were observed after Dex treatment. In addition, considerably elevated marrow adiposity (increased by 159.9%) was found in Dex + control IgG treated rats in comparison to saline control. Thus, the osteoblastogenic differentiation potential of MSCs appeared to be suppressed at the expense of the augment of adipogenesis. As stated above, Wnt/β-catenin signaling plays an essential role in osteogenesis/adipogenesis switch (Georgiou et al., 2012a). Thus, the attenuated Wnt signaling is highly suggested to be associated with the observed bone loss and marrow adiposity in the current Dex treatment model.

Potential correlation amongst chondrocytes/osteocyte apoptosis, CXC12 induction, and chondroclasts/osteoclast recruitment following Dex treatment

As stated above, obvious structural changes in the growth plate and metaphysis were found after Dex treatment, which are associated with induced chondrocyte/osteocyte apoptosis and enhanced chondroclast/osteoclast recruitment and activity. Despite being poorly understood,

the observed tight coupling between the accumulation of apoptotic chondrocytes/osteocytes and the increase in recruitment of chondroclasts/osteoclasts indicated a specific intercellular crosstalk among skeletal cells in response to Dex treatment. Since CXCL12 has been identified to have the most prominent induction (among all chemokines) in Dex-induced apoptotic chondrocytes/osteocytes and CXCL12 derived from these apoptotic cells can promote osteoclast precursor cell migration and osteoclast formation *in vitro* (Tang et al., 2016) (see Chapters 2 and 3), CXCL12 is considered to have the potential to serve as the particular molecular linker related to various aspects of Dex-induced effects on skeletal cell regulation. Thus comprehensive analyses of chondrocyte/osteocyte apoptosis, CXC12 induction, and chondroclast/osteoclast recruitment were performed in the current *in vivo* rat Dex treatment model. As expected, Dex treatment caused a significantly augmented density of apoptotic chondrocytes in the current study. Meanwhile, the majority of apoptotic chondrocytes were detected at the bottom of hypertrophic zone. In addition, RT-PCR analysis showed CXCL12 expression levels are significantly upregulated in growth plate after Dex treatment. In particular, the $CXCL12^{+}$ staining was extensively observed at the bottom rows of chondrocyte columns, principally close to or along the growth plate/metaphysis transitional zone, which strongly suggest CXCL12 induction occurs at apoptotic chondrocytes of the growth plate of treated rats. Similarly, there was also an induction of CXCL12 observed in metaphyseal bone (despite being statistically insignificant in the current RT-PCR gene expression study with the whole bone RNA extract), our *in vivo* studies also revealed the corresponding and significant alterations in the population of apoptotic osteocytes within lacunae expressing CXCL12 upon Dex treatment, suggesting the correlation between osteocyte apoptosis and CXCL12 induction after Dex treatment. Furthermore, the considerably induced chondrocyte/osteocyte apoptosis was found to be accompanied with an increase in $TARP^{+}$ chondroclasts/osteoclasts following Dex treatment.

Based on these findings, chemokine CXCL12, derived from Dex treatment induced-apoptotic chondrocytes/osteocytes, appeared to have the potential to act as a compelling determinant for chondroclast/osteoclast recruitment and activity, which causes significant cartilage/bone loss, leading to the bone growth defects in growing long bones.

Potential role of CXCL12 blockade with an anti-CXCL12 neutralizing antibody in preventing Dex treatment-induced damaging effects on skeleton

Previous studies have illustrated that CXCL12 is associated to numerous skeletal cell regulation pathways, and particularly it has been shown to play an essential role in osteoclast recruitment and differentiation (Gronthos and Zannettino, 2007). It is evident that CXCL12 could trigger the induction of matrix metalloprotease-9 (MMP-9), an enzyme mandatory for the penetration through basal lamina barrier, thereby promoting the recruitment and migration of circulating osteoclast precursors (Yu et al., 2003). In addition, CXCL12 has been shown to have the potential to expand precursor cell pool and stimulate multinucleated cell fusion, size and TRAP activity during the stage of osteoclastogenesis (Wright et al., 2005). In the current study, since Dex-induced cartilage/bone loss was considered to be potentially mediated by CXCL12, loss-of-function studies of CXCL12 were performed to further investigate and verify its roles in bone growth defects following Dex treatment. Firstly, there were no statistically significant differences in growth plate and metaphysis structural changes were noted between the saline-treated rats with anti-CXCL12 neutralizing antibody and normal control IgG-supplementation, indicating I.P. injection of the anti-CXCL12 neutralizing antibody at 1 mg/kg t.i.w. (three times per week) for 2 weeks does not affect the major variables of the bone growth unit in healthy rats. Interestingly, it was also found the Dex-induced obvious reductions in growth plate thickness, primary spongiosa height and trabecular bone volume could be significantly, at least partially, prevented or restored to the

normal control level (increased to 116.0%, 108.7 %, and 139.5%, respectively, compare to Dex + IgG group) by the supplementation of anti-CXCL12 neutralizing antibody, which suggests that anti-CXCL12 neutralizing antibody is a potential candidate to prevent or control cartilage/bone loss during Dex treatment. Although CXCL12 is evident to be associated with the differentiation of osteoblasts, apoptosis of skeletal cells and adipocytogenesis in bone marrow stromal cells (Georgiou et al., 2012c; Hosogane et al., 2010; Wei et al., 2006), supplementary anti-CXCL12 neutralizing antibody tended to exert no or only slight effects on osteoblast activity, chondrocyte/osteocyte apoptosis and marrow adiposity under the current experimental settings. However, markedly inhibited chondroclast/osteoclast recruitment (densities decreased to 56.2% and 44.0%, respectively, in comparison to control IgG) was observed after Dex treatment in the presence of anti-CXCL12 neutralizing antibody. Consistently, our RT-PCR analyses also showed that anti-CXCL12 neutralizing antibody appeared to have the capacity to maintain the RANKL/OPG ratio, the well-known determinant for osteoclastogenesis, within the normal control levels following Dex treatment. In addition, *ex vivo* osteoclast formation studies revealed that anti-CXCL12 neutralizing antibody supplementation could considerably suppress Dex-induced enhanced osteoclastogenic differentiation potential. Collectively, these findings strongly suggested that anti-CXCL12 neutralizing antibody is a potent osteoclastogenesis inhibitor, thereby preserves endochondral ossification and particularly prevents excessive bone resorption after Dex treatment.

CXCL12 is known to exert biological effects through binding with its unique transmembrane receptor CXCR4; therefore further studies are required to further investigate the potential roles of CXCL12-CXCR4 axis, and the potential effects of CXCR4 receptor inhibitor in preventing, Dex-induced damaging effects on skeleton. In addition, the current *in vivo* Dex

treatment model was established in healthy rats. However, Dex is one of the most commonly and intensively used agents in childhood oncology. In an effort to further address the underlying mechanisms of Dex treatment-related bone defects, additional rat Dex treatment models including cancer-bearing models are also required in future work.

Conclusion

In summary, obvious structural changes in the growth plate and metaphysis were observed following Dex treatment. These were demonstrated to be partially associated with induced chondrocyte/osteocyte apoptosis and enhanced chondroclast/osteoclast recruitment and activity, which were potentially molecularly linked by the chemokine CXCL12 induced by the apoptotic chondrocytes/osteocytes. Furthermore, blocking the function of CXCL12 by the supplementation of anti-CXCL12 neutralizing antibody could significantly preserve bone mass and bone growth after Dex treatment, principally by the inhibition of aggravated bone resorption. These findings provide novel insights into molecular mechanisms underlying Dex treatment-induced adverse effects on skeleton, and will contribute to the development of therapeutic targets for preventing or treating bone growth defects in children receiving Dex therapy.

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Disclosure

The authors declared that they have no conflict of interests to this work.

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CHAPTER FIVE

General discussion, Conclusion, and Future directions

5.1 General Discussion

Cancer chemotherapy-induced bone growth defects in childhood

Bone is a metabolically active tissue and bone growth is a multiple-step process consisting of diverse morphological and biochemical changes during fetal and postnatal development (van der Eerden et al., 2003). Since peak growth is achieved in late puberty, childhood is an extremely vital period for bone growth and development (Mikkola et al., 2017). However, the intensive or prolonged use of chemotherapy in childhood has been shown to cause severe adverse effects on the skeleton, which could persist for a long term or even into adulthood (Fan et al., 2011b; Su et al., 2017; te Winkel et al., 2014). For example, although acute lymphoblastic leukemia (ALL), the most common childhood malignancy, has an over 80% of survival rate following chemotherapeutic agent treatment involving methotrexate (MTX) and dexamethasone (Dex) (Mitchell et al., 2009), the pediatric ALL patients and survivors have been observed to be associated with the significantly increased prevalence of bone growth defects such as bone growth arrest, low bone loss and associated fractures during and/or after chemotherapy (Viana and Vilela, 2008), which places a tremendous social, emotional and economic burden to them and their families (Heath et al., 2006).

It is evident that chemotherapy could cause the severe disruptions to the regulation and coordination of the skeletal cells residing in the growth plate cartilage, bone and marrow microenvironment, leading to the bone growth defects (Xian et al., 2007). However, the underlying cellular and molecular mechanisms still remain largely unknown. It is unclear whether the induced disruptions to steady-state skeletal cellular homeostasis occur dependently or whether there exist interdependent relationships between them. Due to the lack of preventive strategies and specific treatments to minimize chemotherapy-induced

adverse skeletal effects, it has become more urgent to further understand the pathogenesis and to develop novel, safe, and cost-effective therapeutic strategies preserving bone growth during/after chemotherapy in childhood.

Potential roles of cytokine/chemokines to act as the molecular linker between induced chondrocyte/osteocyte apoptosis and enhanced chondroclast/osteoclast recruitment and activity after MTX or Dex therapy

It is beyond the scope of the current project to detail all categories of chemotherapeutic agents and regimens. Methotrexate (MTX) and Dexamethasone (Dex), the two most commonly and intensively used agents in childhood oncology, particularly in the treatment of ALL (Crofton et al., 2000), have been selected for further investigation in this project. MTX is an anti-metabolite that inhibits the enzyme dihydrofolate reductase (Minaur et al., 2002), while Dex is a potent member of the glucocorticoid class of steroids which is with essential anti-inflammatory and immunosuppressive properties (Crofton et al., 2000). Both of them have been prescribed at high-doses in pediatric malignancy treatment, particularly in the treatment of ALL (Veerman et al., 2009). As stated above, characteristic cellar disruption s have been observed following MTX or Dex treatment, such as induced chondrocyte/osteocyte apoptosis accompanied by enhanced chondroclast/osteoclast recruitment and activity, which is associated with the uncontrolled cartilage and/or bone loss and the impairment of bone growth (Fan et al., 2011a; Olney, 2009; Shandala et al., 2012; Zaman et al., 2012). Interestingly, previous studies have also found a similar coupling between the accumulation of apoptotic osteocytes and the increase in recruitment of osteoclasts driven by various external factors (Bellido, 2014; Kennedy et al., 2012; Plotkin, 2014), suggesting an underlying intercellular crosstalk tightly coordinating skeletal cell regulations in response to external influences including chemotherapy.

Recently, the interplay between skeletal and immune systems has been gradually highlighted. Accumulating evidence has revealed their overlapping regulatory mechanisms by which skeletal and immune cells shared the regulators in common, including a subset of cytokine(s) and chemokine(s) (Caetano-Lopes et al., 2009). Cytokines are a broad category of messenger proteins for specific biological functions, while chemokines are a large family of small chemotactic cytokines, both of which are well-known to be crucial for the migration, formation and regulation of immune cells (Caetano-Lopes et al., 2009). Some specific cytokine(s) and chemokine(s) (e.g. CXCL12) have been demonstrated to participate in multiple skeletal cell regulation pathways and play essential roles in the coordination of trafficking, differentiation, activity and apoptosis of skeletal cells in physiological and pathological conditions (Gilchrist and Stern, 2015). In particular, the increased expression of cytokine(s) and chemokine(s) in bone has been shown to play a critical role in enhanced osteoclastogenesis (Gronthos and Zannettino, 2007; King et al., 2012; Wright et al., 2005; Yu et al., 2003). Therefore, a specific subset of cytokine(s) and chemokine(s) may be ideal candidates for the investigation to elucidate the molecular link between skeletal cell apoptosis and osteoclast differentiation following chemotherapy.

Feasible in vitro models for mature chondrocyte/osteocyte MTX and/or Dex treatment studies

Thus in the current study, it was hypothesized that MTX ± Dex treatment could induce chondrocyte/osteocyte apoptosis and these apoptotic cells in turn trigger the induction of specific cytokine(s) and/or chemokine(s) to promote chondroclast/osteoclast recruitment and formation, resulting in the uncontrolled cartilage/bone loss. To address this hypothesis, ATDC5 chondrogenic cell line, the mouse embryonic carcinoma-derived cell line on the basis of chondrogenic potentials (Shukunami et al., 1997), and Immortomouse/Dmp1-GFP (IDG)-

SW3 osteoblastic/osteocytic cell line, generated by crossing the immortomouse with the Dmp1-GFP transgenic mouse (Woo et al., 2011), were employed to develop the *in vitro* chondrogenesis and osteogenesis models. Previously, it has been shown that MTX or Dex treatment could significantly induce chondrocyte apoptosis, which is principally in terminally differentiated hypertrophic chondrocytes (Chrysis et al., 2003; Xian et al., 2007; Zaman et al., 2012); and while osteocytes are the terminally differentiated osteoblastic cells, IDG-SW3 cell line has the potential to recapitulate the differentiation process from late osteoblasts to mature osteocytes (Woo et al., 2011). Thus, to find a suitable time point for the MTX ± Dex treatment studies with cultured mature chondrocytes/osteocytes, it is necessary to appropriately assess the processes of differentiation of ATDC5 chondrogenic cells and IDG-SW3 osteocytic cells in culture. In the current study, time-course studies studies by using Alcian blue staining, immunofluorescence analyses and qRT-PCR analyses for the selective molecular markers confirmed the suitability of ATDC5/IDG-SW3 cell line as the model for forming mature chondrocytes/osteocytes. For the subsequent *in vitro* parts of the study in this project, MTX ± Dex treatment was therefore conducted during the late stage of chondrogenic/oestrogenic differentiation of both cell lines to examine the treatment effects on the apoptosis induction of mature chondrocytes/osteocytes and the expression of cytokines/chemokines.

Chondrocyte/osteocyte apoptosis induced by MTX and/or Dex treatment in vitro

Apoptosis is the default fate of most nucleated cells including chondrocytes and osteocytes (Green, 1998). Although the normal rate of chondrocyte/osteocyte apoptosis has been confirmed to be vital for skeletal health (Jilka et al., 2014; Mackie et al., 2011), the adverse effects of MTX and Dex therapy on the skeleton are induced in part by an increase in chondrocyte/osteocyte apoptosis, which is associated with thinning of the growth plate and

bone growth impairment (Spreafico et al., 2008; Weinstein, 2011; Xian et al., 2008; Xian et al., 2007; Zaman et al., 2012). In the current study, ATDC5 and IDG-SW3 cells, which are in the mature chondrocyte and mature osteocyte stage, respectively, were treated with MTX at 10^{-5}M and/or Dex at 10^{-6}M *in vitro*, which has been shown to cause cell apoptosis as previously described (Kogianni et al., 2004; Shandala et al., 2012). Significantly enhanced apoptosis induction was observed in both ATDC5 and IDG-SW3 cells in response to MTX ± Dex treatment as shown by immunofluorescence labelling for cleaved caspase-3, a key mediator of apoptosis (Budihardjo et al., 1999). In addition, it has been well established that cell apoptosis is mainly mediated by two major signaling pathways in mammalian cells: the intrinsic and extrinsic pathways, which are determined by regulatory genes Bax/Bcl-2 and Fas/Fas-L respectively (Danial and Korsmeyer, 2004). Consistently, obvious changes in mRNA expression levels of the above apoptosis regulatory genes were found following MTX ± Dex treatment by using real-time qRT-PCR analyses. In Brief, the current findings indicated Dex-induced chondrocyte apoptosis may particularly be through the intrinsic Bax/Bcl-2 pathway, while chondrocyte apoptosis induced by MTX treatment may be principally determined by the extrinsic Fas/Fas-L pathway. On the other hand, it was also suggested that MTX ± Dex-induced osteocyte apoptosis is regulated through both the intrinsic pathway and the extrinsic pathway in the present *in vitro* model. Collectively, immunofluorescence and qRT-PCR analyses showed that MTX ± Dex treatments do cause significant induced apoptosis of chondrocytes and osteocytes. Nevertheless, further studies are still required to investigate the mechanisms involved in chondrocyte/osteocyte apoptosis after MTX and/or Dex treatment.

Enhanced chondroclast/osteoclast recruitment and activity following MTX and/or Dex treatment in vitro

During normal endochondral ossification, osteoclasts identified within growth plate/metaphysis transitional zone (also called as chondroclasts) are specifically involved the resorption of calcified cartilage (Vu et al., 1998) and are known to play a critical role in endochondral ossification (Mackie et al., 2011). Approximately two-thirds of cartilage matrix is actively degraded by chondroclasts, whilst the cartilaginous remnants acts as a template for further bone formation (Ballock and O'Keefe, 2003). In addition, osteocytes are known to play a crucial role in the formation and activities of osteoblasts and osteoclasts, and therefore coordinating the ongoing balance between bone resorption and formation during bone remodeling (Nakashima et al., 2011; O'Brien et al., 2013; Xiong et al., 2011). Intensive use of MTX or Dex treatment has been shown to cause both direct and indirect interruption of skeletal homeostasis, resulting in severe adverse effects on skeleton (Canalis et al., 2007; Xian et al., 2007). Despite being poorly understood, induced chondrocyte/osteocyte apoptosis accompanied by enhanced chondroclast/osteoclast recruitment and activity have been observed following MTX or Dex treatment (Fan et al., 2011a; Olney, 2009; Shandala et al., 2012; Zaman et al., 2012), suggesting apoptotic chondrocytes/osteocytes have the potential to express some regulatory factors to stimulate chondroclast/osteoclast recruitment, formation and activity after treatment, which results in the uncontrolled cartilage/bone loss.

It has been well-established that osteoclast precursors are selectively recruited and thereafter differentiate into multinucleated and activated osteoclasts during the process of bone resorption (Kikuta and Ishii, 2013). Thus, mouse RAW 264.7 osteoclast precursor cells were subjected to *in vitro* osteoclast precursor migration and formation assays to address the potential role of chondrocyte/osteocyte apoptosis caused by MTX and/or Dex treatment in

osteoclastic cell recruitment and differentiation. The current study treated osteoclast precursor cells with conditioned media from MTX ± Dex-treated vs -untreated ATDC5 chondrogenic and IDG-SW3 osteocytic cells. It was found that conditioned medium from treated chondrocytes/osteocytes has the capacity to considerably enhance osteoclast precursor migration and formation, suggesting some potent osteoclastogenesis-stimulating factors have been derived from apoptotic chondrocytes/osteocytes after MTX and/or Dex treatment in the present *in vitro* model.

CXCL12 exhibited the most prominent induction in MTX and/or Dex treatment-induced apoptotic chondrocytes/osteocytes

As stated above, a number of cytokines and chemokines have been demonstrated to act as the essential mediators for chemotaxis, differentiation, function and survival of skeletal cells, and particularly involved in enhanced osteoclastogenesis (Gronthos and Zannettino, 2007; King et al., 2015; Wright et al., 2005; Yu et al., 2003). Specific cytokine(s)/chemokine(s) could be considered as an ideal candidate serving as a potential intercellular cross-talk molecular link within the skeletal microenvironment. To systematically investigate the effects of MTX and/or Dex treatment on the expression of the cytokine/chemokine family in chondrocytes/osteocytes, a specific PCR array containing 84 major known cytokines and chemokines was conducted. Consistent with the findings in previous studies (Andreas et al., 2009; Borzi et al., 2004; Young et al., 2001), a number of cytokines and chemokines (e.g. CXCL12, CCL2, IL-17f and TNF-α) were observed to achieve elevated expression after MTX and/or Dex treatment. Among all the examined candidates, chemokine CXCL12, a member of the CXC family of chemokines, was found to exhibit the most prominent induction in each treatment group, suggesting apoptotic chondrocyte/osteocyte-derived CXCL12 may be the major player in the current *in vitro* model. The upregulation of CXCL12

was further supported by conformational experiments that both its mRNA expression levels and the protein concentrations in conditioned medium from MTX ± Dex-treated chondrocytes/osteocytes were notably increased.

CXCL12 derived from apoptotic chondrocytes/osteocytes is one of the determinants for osteoclast recruitment and differentiation following MTX and/or Dex treatment in vitro

According to these findings, CXCL12 was selected as the candidate of interest and the current study subsequently investigate the potential role of apoptotic chondrocyte/osteocyte-derived CXCL12 in osteoclast precursor cell migration and osteoclast formation *in vitro.* Thus, the above migration and formation assay in combination with blocking experiments were performed to further identify whether the CXCL12 functional blockage has an influence on the observed results. In the migration assay, it was found that both exogenous recombinant CXCL12 protein and the CXCL12 contained in the conditioned medium from MTX, Dex, or their combination-treated chondrocytes/osteocytes have the capacity to stimulate osteoclast precursor cell migration, while this migration promotion from the conditioned medium of treated cells could considerably inhibited by the presence of the neutralizing anti-CXCL12 antibody in each treatment group. On the other hand, in the formation assay, it was observed that RANKL is mandatory for osteoclast differentiation in culture and that exogenous recombinant CXCL12 protein has no or only a weak capacity to trigger osteoclast differentiation. Interestingly, in the presence of RANKL, recombinant CXCL12 protein as well as MTX ± Dex-treated cell conditioned medium were shown to be capable of significantly stimulating osteoclast formation in comparison to conditioned medium from untreated cells. Consistent with the findings in the migration assay, this stimulating effect was also considerably repressed after CXCL12 blockade by using the anti-CXCL12 neutralizing antibody among all the treatment groups. Collectively, these findings suggested

that, aside from the well-known RANKL pathway, CXCL12 derived from apoptotic chondrocytes/osteocytes following MTX ± Dex treatment is one of the determinants for osteoclastogenesis *in vitro*, which exerts direct and indirect effects in the recruitment, formation and function of osteoclasts.

Dex treatment induced the dysfunction of growth plate in vivo

Since the above *in vitro* studies have shown CXCL12 achieves the most prominent induction after MTX ± Dex treatment, and the induced skeletal cell apoptosis and enhanced osteoclast differentiation could be molecularly linked by CXCL12, the next step of the current project was using a Dex treatment + CXCL12 functional blockage model in young rats to investigate Dex-induced structural, cellular and molecular damages within bone and marrow microenvironment, and to examine any potential protective effects of anti-CXCL12 neutralizing antibody supplementation. In the current *in vivo* rat Dex treatment model which is aim to mimic the clinical regimen for acute ALL maintenance treatment as described (Veerman et al., 2009), similar to the findings in the previous *in vitro* studies, chondrocyte apoptosis was found to be strikingly induced upon Dex treatment. The apoptotic chondrocytes were expressively detected in the growth plate by using Hoechst dye and haematoxylin staining, which is mainly within the hypertrophic zone. Meanwhile, considerably augmented recruitment of chondroclasts was also observed following Dex treatment. In addition, histomorphometric measurements revealed that the Dex treatment in the absence of anti-CXCL12 neutralizing antibody supplementation causes a significant reduction in growth plate thickness and adjacent primary spongiosa height, which is at least in part attributed to enhanced chondrocyte apoptosis and chondroclast recruitment, and therefore caused the deficiency of calcified cartilage scaffold for subsequent bone formation, resulting in the suppression of longitudinal growth and bone mass accumulation.

Dex treatment induced the disruption of bone homeostasis

Under physiological circumstances, there is a balance between the coupled bone formation and resorption (Rosen et al., 2009). However, in situations such as glucocorticoid therapy, this ongoing balance is disrupted, resulting in the impaired bone remodeling and uncontrolled bone loss (Hardy and Cooper, 2009). In the current study, mirroring to the decreased growth plate and primary spongiosa heights, a sharp reduction in trabecular bone volume within secondary spongiosa was observed in Dex + control IgG treated-rats in comparison to saline controls, suggesting significant bone loss was induced after Dex treatment. Interestingly, compared with the reduced trabecular density (decreased to 73.3%), Dex treatment appeared to have more significant effects on the alterations in trabecular thickness (decreased to 46.9%) and trabecular spacing (increased to 215.5%), indicating the induced bone loss is mainly owing to excessive bone resorption rather than inhibited bone formation in the present *in vivo* model.

Although the mechanisms still remain unclear, previous studies have shown intense use of Dex treatment could enhance osteoclast differentiation, which is associated with the upregulation of RANKL (Hofbauer and Rauner, 2009) and the inhibition of OPG (Sasaki et al., 2002), leading to promoted bone resorption (Jia et al., 2006). In the current study, the RANKL/OPG ratio, which is the determinant for osteoclastogenesis (Weinstein, 2012), was observed to be notably upregulated in the metaphysis after Dex treatment by using RT-PCR gene expression analyses. Histomorphometric analyses also showed that the densities of osteoclast in the metaphyseal bone were considerably increased in response to Dex treatment. In addition, the impact of Dex treatment on osteoclastogenic differentiation potential of the bone marrow cells was examined by an *ex vivo* osteoclast formation assay. The obviously augmented densities of TRAP-positive multinucleated osteoclasts were found following Dex treatment, suggesting the significantly improved osteoclastogenic differentiation potential

caused by Dex treatment, which could result in the elevated osteoclast density on the bone surface. Collectively, enhanced osteoclast formation and activity were greatly induced upon Dex treatment, contributing towards aggravated bone resorption.

Aside from enhanced osteoclastogenesis, Dex treatment has been demonstrated to cause a negative effect on osteoblast formation (Bouvard et al., 2010). However, no statistically significant differences in osteoblast densities along trabecular bone surface between saline and Dex treatment groups were noted in the current study, suggesting Dex treatment appears to have insignificant or slight effects on osteoblast densities under circumstances, which is inconsistent with the previously observed alterations in trabecular bone structure and volume. Gene expression studies on key osteoblastogenesis-related transcription factors in the metaphysis were therefore carried out to further examine the treatment effects of Dex on bone formation. Consistent with the change pattern of the osteoblast density, an observable but insignificant decrease in expression of metaphyseal Osx (the molecular marker for the late stage of osteoblastogenic differentiation) and OCN (the most abundant non-collagenous protein of bone matrix) was noted after Dex treatment. However, the mRNA expression levels of Runx2 (the molecular marker for the early stage of osteoblastogenic differentiation) were strikingly decreased upon Dex treatment, indicating the depressed osteoblast differentiation. In addition, the effects of Dex treatment on osteogenesis potential of the bone marrow stromal cells was examined by an *ex vivo* mineralization assay. Conspicuously observed mineralized matrix nodules and higher-intensity of Alizarin-Red staining were found from the saline control groups but less so in Dex-treated rats, which suggest osteoblastogenesis and mineralization potential are markedly inhibited in the bone marrow after Dex treatment in culture. Moreover, it is well-established that canonical Wnt/β-catenin signalling is essential in promoting osteoblast differentiation but suppressing adipogenic differentiation in the bone marrow (Hardy and Cooper, 2009; Macsai et al., 2008), which

could be attenuated by Dex administration through up-regulated expression of Wnt antagonists such as Dkk-1 and SFRP-1, leading to the ultimate inhibition of osteoblast differentiation (Bouvard et al., 2010; Georgiou et al., 2012a). In the current study, despite the slight increasing trend in SPRF-1 expression, the mRNA expression levels of Dkk-1 and SOST were markedly upregulated after Dex treatment, suggesting a potential defect in canonical Wnt/β-catenin signaling pathway which may have played a role in mediating Dex treatment-induced osteogenic differentiation. Taken together, it was shown that Dex treatment indeed exerts an inhibitory effect on bone formation, which was induced by the significant negative effect on osteoblast formation and function.

Dex treatment induced the alteration in bone marrow adipocyte population

It has been illustrated that bone marrow MSCs are pluripotent progenitor cells which can differentiate into various lineages, including osteogenic and adipogenic lineages (Pittenger et al., 1999). It is also evident that intensive Dex treatment can cause a significant switch in MSC commitment from osteogenesis to adipogenesis from the common precursor shared by osteoblasts and adipocytes (Canalis et al., 2007). To investigate the treatment effects on adipogenesis in the bone marrow microenvironment, the density of adipocyte was quantified at the lower secondary spongiosa. Dex treatment was revealed to cause a significant increase in adiposity. In addition, as stated above, Wnt/β-catenin signaling plays an important role in osteogenesis/adipogenesis switch (Georgiou et al., 2012a). In the current study, aside from the downregulated expression levels of Runx2, the markedly upregulated expression levels of C/EBPα (an adipogenesis transcription factor) were observed after Dex treatment. Thus, these findings suggested the augment of adipogenesis was induced by Dex treatment, while the osteoblastogenic differentiation potential of MSCs appeared to be suppressed.

Potential correlation amongst chondrocytes/osteocyte apoptosis, CXC12 induction, and chondroclasts/osteoclast recruitment following Dex treatment

Thus our Dex treatment rat model observed obvious structural changes in the growth plate and metaphysis, which are associated with induced chondrocyte/osteocyte apoptosis accompanied by enhanced chondroclast/osteoclast recruitment and activity. With our *in vitro* observation of the most prominent induction of CXCL12 in apoptotic chondrocytes and osteocytes and as it has been illustrated that CXCL12 plays an essential role in osteoclast recruitment and differentiation (Gronthos and Zannettino, 2007), using the rat Dex treatment model, the current study subsequently investigated the co-location relationship between skeletal cell apoptosis and CXCL12 induction, to further explore the potential role of CXCL12 in serving as the molecular linker related to various aspects of Dex-induced effects on skeletal cell regulation.

As expected, CXCL12 mRNA expression levels were found to be significantly upregulated in growth plate after Dex treatment. In particular, immunohistochemistry analysis showed that the $CXCL12^{+}$ staining was extensively observed at the bottom of chondrocyte columns, principally close to or along the growth plate/metaphysis transitional zone, where the majority of apoptotic chondrocytes were detected. These findings strongly suggested that CXCL12 induction in the growth plate was induced after treatment, principally occurring at apoptotic chondrocytes. Similarly, RT-PCR analysis also revealed a upregulation of CXCL12 expression (despite being statistically insignificant in the current gene studies with the whole bone RNA extract) in metaphyseal bone of Dex-treated rats. Meanwhile, in comparison to saline controls, a significant increase in the densities of apoptotic osteocytes within bony trabeculae in metaphysis was observed by using ISNT labelling following Dex treatment. The corresponding and significant alterations in the population of apoptotic osteocytes within

lacunae expressing CXCL12 upon Dex treatment were also observed, indicating the correlation between osteocyte apoptosis and CXCL12 induction in our *in vivo* studies.

Thus, due to the observed correlation amongst chondrocytes/osteocyte apoptosis, CXC12 induction, and chondroclasts/osteoclast recruitment following Dex treatment, chemokine CXCL12, derived from Dex treatment induced-apoptotic chondrocytes/osteocytes, appeared to act as a compelling determinant for chondroclast/osteoclast recruitment and activity, which causes significant cartilage/bone loss in growing long bones. Consistently, previous studies have shown that CXCL12 could trigger the induction of matrix metalloprotease-9 (MMP-9), an enzyme mandatory for the penetration through basal lamina barrier, thereby promoting the recruitment and migration of circulating osteoclast precursors (Yu et al., 2003). CXCL12 has also been demonstrated to have the potential to expand precursor cell pool and stimulate multinucleated cell fusion, size and TRAP activity during the stage of osteoclastogenesis (Wright et al., 2005).

Potential role of CXCL12 blockade with an anti-CXCL12 neutralizing antibody in preventing Dex treatment-induced damaging effects on skeleton

As the next step, the potential role of CXCL12 blockade with an anti-CXCL12 neutralizing antibody in preventing Dex treatment-induced damaging effects on skeleton was thereafter investigated. In saline-treated control rats, there were no statistically significant differences in growth plate and metaphysis structures between the anti-CXCL12 neutralizing antibody and normal control IgG supplementation groups, indicating the administration of the anti-CXCL12 neutralizing antibody does not affect the key variables of the bone growth unit in healthy rats. Interestingly, it was found that these Dex-induced reductions in growth plate thickness, primary spongiosa height and trabecular bone volume could be considerably, at

least partially, prevented or restored to the normal control levels by the supplementation of anti-CXCL12 neutralizing antibody, which suggests that anti-CXCL12 neutralizing antibody is a potential candidate to prevent or control cartilage/bone loss during Dex treatment.

Although CXCL12 is evident to participate in multiple skeletal cell regulation pathways (Georgiou et al., 2012b; Hosogane et al., 2010; Wei et al., 2006), supplementary anti-CXCL12 neutralizing antibody tended to exert no or only slight effects on osteoblast activity, chondrocyte/osteocyte apoptosis and marrow adiposity under the current experimental settings. On the other hand, Dex-induced significantly enhanced chondroclast/osteoclast recruitment and the RANKL/OPG expression ratio were attenuated significantly in Dex-treated rats also treated with the anti-CXCL12 neutralizing antibody. Consistently, ex vivo osteoclast formation studies revealed that anti-CXCL12 neutralizing antibody supplementation could considerably suppress Dex-induced osteoclastogenic differentiation potential. Collectively, these findings suggested that anti-CXCL12 neutralizing antibody is a potent osteoclastogenesis inhibitor, thereby have the potential to preserve endochondral ossification and particularly prevent excessive bone resorption after Dex treatment.

5.2 Conclusions

In summary, `the current PhD project employed *in vitro* MTX and/or Dex treatment models in cultured mature ATDC5 chondrocytes and mature IDG-SW3 osteocytes to investigate whether MTX ± Dex-treatment-induced apoptotic chondrocytes/osteocytes secrete specific cytokine(s)/chemokine(s) with the capacity to stimulate osteoclast recruitment and differentiation. Chemokine CXCL12 was identified to exhibit the most prominent induction in the MTX ± Dex-treated chondrocytes/osteocytes. In particular, induced

chondrocyte/osteocyte apoptosis and enhanced RAW264.7 osteoclast precursor migration and their osteoclast formation were observed to be potentially or at least partially linked molecularly by CXCL12 induction *in vitro*. These findings suggested that CXCL12 derived from apoptotic chondrocytes/osteocytes following MTX and/or Dex treatment is a novel osteoclastogenesis stimulating factor, which is associated with aggravated cartilage or bone loss, leading to the bone growth defects.

A rat Dex treatment in combination with CXCL12 blockade model was subsequently utilized to further confirm the role of CXCL12 in Dex-induced bone growth defects and to investigate the potential protective effects of anti-CXCL12 neutralizing antibody supplementation. It was shown that Dex treatment indeed causes obvious structural changes in the growth plate and metaphysis, which is associated with induced chondrocyte/osteocyte apoptosis, enhanced chondroclast/osteoclast recruitment, stimulated osteoclastogenic differentiation potential, marginally inhibited osteoblastogenic differentiation potential and promoted marrow adiposity. However, blocking the function of CXCL12 by the supplementation of anti-CXCL12 neutralizing antibody could significantly preserve bone mass and bone growth after Dex treatment, which is principally by the inhibition of aggravated cartilage and bone loss.

The date from the current PhD project provides novel insights into molecular mechanisms underlying MTX ± Dex treatment-induced adverse effects on skeleton and allowed us to grasp a better understanding of the potential role of chemokine CXCL12 in skeletal homeostasis. Specifically, we demonstrated that CXCL12 is a molecular link between Dex treatment-induced skeletal cell apoptosis and resorptive cell recruitment and bone loss following Dex treatment, and that blocking CXCL12 function can attenuate adverse skeletal effects caused by Dex treatment. Due to the current lack of adjunct preventative treatments

for minimizing the chemotherapy- associated adverse skeletal effects, these findings also provide a molecular basis to develop future therapeutic strategies. The supplementation of anti-CXCL12 neutralizing antibody has been shown to be a potential ideal therapeutic approach for preventing or treating bone growth defects in paediatric patients receiving MTX and/or Dex therapy.

5.3 Future Directions

In the current PhD project, both *in vitro* and *in vivo* studies have shown that CXCL12 is a potent osteoclastogenesis stimulating factor in response to chemotherapeutic agent treatment. In particular, it was observed that the potential effects of CXCL12 blockage with the supplementation of anti-CXCL12 neutralizing antibody in preventing cartilage and bone loss after Dex therapy were principally due to the inhibition of Dex-stimulated osteoclast precursor cell recruitment and osteoclastogenic differentiation. Apart from osteoclastic cell formation and activity, CXCL12 tended to exert no or only slight effects on the other aspects of bone homeostasis such as osteoblast activity and marrow adiposity. However, previous studies have shown that CXCL12 is not only strongly associated with osteoclastogenesis, but also participates directly in a wide range of signaling pathways within bone and marrow microenvironment. For example, it has been revealed that CXCL12 has the potential to stimulate osteogenic differentiation of MSCs (Hosogane et al., 2010), particularly in the early stage (Li et al., 2017). CXCL12 was also found to contribute to the expression of VEGF, and the inhibition of CXCL12 could suppress VEGF-dependent angiogenesis, resulting in the impaired endochondral ossification (Grunewald et al., 2006). Therefore, further work is required to systematically clarify the action mechanisms of CXCL12 in bone homeostasis, particularly during/after chemotherapy.

A prominent induction of CXCL12 was found in the growth plate and metaphysis *in vivo* in the present study. Interestingly, elevated serum CXCL12 levels (Georgiou et al., 2012b) were also observed after MTX chemotherapy in rats, which showed a temporal relationship with the increased osteoclastic recruitment and resorption *in vivo* (King et al., 2012). While upregulated serum levels of CXCL12 are demonstrated to be associated with increased osteoclast activity and osteolysis in multiple myeloma patients (Zannettino et al., 2005), further studies are required to investigate the roles of apoptotic chondrocyte/osteocyte-derived CXCL12 or its higher circulation levels in bone loss following cancer chemotherapy.

In addition, MTX and Dex are the two most commonly and intensively used agents in childhood oncology, particularly their combination therapy is the key component of the chemotherapy regimen for ALL maintenance treatment in children (Veerman et al., 2009). Although it has been well-established that intensifying MTX/Dex monotherapy could cause bone defects, it is still unclear whether the skeleton impairments induced by MTX combination therapy with Dex are influenced by the synergistic or antagonistic effects of these two agents, leading to the inability to fully understand the underlying mechanisms and provide effective management strategies. Thus, further studies should be performed to provide the comparative profiles of MTX and/or Dex treatment in causing the skeletal defects. On the other hand, the therapeutic potential of CXCL12 blockade with the supplementation of anti-CXCL12 neutralizing antibody in preventing cartilage/bone loss has been investigated by using Dex treatment model. Similar studies are required to further explore the potential role of CXCL12 function blockage in bone growth defects during/after MTX ± Dex treatment.

Furthermore, it has been demonstrated that CXCL12 exerts biological effects through binding

with its seven-transmembrane G protein-coupled receptor CXCR4 (Liekens et al., 2010). CXCL12/CXCR4 axis has been proven to act as an essential communication bridge within bone and marrow microenvironment (Georgiou et al., 2012b; Moll and Ransohoff, 2010; Wei et al., 2012). In the current project, it was found that induction of CXCL12 is tightly associated with chemotherapy-induced adverse effects on skeleton. Therefore, to minimize these adverse skeletal effects, a potential therapeutic strategy is to disrupt CXCL12/CXCR4 interactions, which could in turn prevent downstream effects and therefore gain therapeutic benefits. Recently, more attention has been paid to the antagonists of CXCR4, which are analogues and peptides designed to interfere with CXCR4 and prevent its activation (Sun et al., 2010). Multiple CXCR4 inhibitors such as bicyclams (AMD3100), TN14003 and ALX40-4C, which selectively target the amino-terminal region of the CXCL12, have been developed and confirmed to be with a low level of toxicity (Donahue et al., 2009; Hojo et al., 2007). Thus, although a greater understanding of the role of CXCL12 and CXCR4 in modulating skeletal cell regelation is required, CXCL12/CXCR4 axis is a promising actionable therapeutic target using CXCR4 inhibitors for chemotherapy-induced bone growth defects.

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APPENDIX

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Chemokines: a potential molecular link between chondrocyte apoptosis and osteoclast migration and formation following dexamethasone therapy

Qian Tang

University of South Australia, Australia

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Introduction: Glucocorticoid therapy is known to cause growth plate chondrocyte damage, bone growth arrest, bone resorption, and bone loss, for which the mechanisms remain unclear. The current study aims to identify whether apoptotic chondrocytes secrete factors that may stimulate osteoclast differentiation. It was hypothesised that dexamethasone (Dex) will induce chondrocyte apoptosis, causing increased secretion of chemokines with the capacity to promote osteoclast recruitment and formation.

Subjects and Methods: The current study utilized an *in vitro* Dex treatment model in ATDC5 chondrogenic cells and examined treatment effects on molecular marker expression, induction of apoptosis, and expression of chemokines and receptors in differentiated Dex-treated chondrocytes. In addition, ability of promoting osteoclast differentiation in RAW264.7 osteoclast precursor cells was assessed for conditioned media taken from Dex-treated or -untreated chondrocytes.

Results: In a time course of chondrogenic culture, the increased type II and X collagen expression suggests the dynamic chondrocyte differentiation. The significantly enhanced increased density of apoptotic cells as observed by Hoechst dye staining as well as markedly increased expression level of Fas-L as examined by real-time quantitative RT-PCR suggests increased chondrocyte apoptosis following Dex treatment. Meanwhile, chemokine PCR array analysis of Dex-treated or -untreated chondrocytes identified up-regulation of various chemokines in treated chondrocytes. Chemokine SDF-1 was demonstrated to be the chemokine with the highest induction following treatment, and its induction was also supported by conformational RT-PCR. Furthermore, the SDF-1 protein level was also increased as assayed by ELISA in the conditioned medium of Dex-treated chondrocytes. Moreover, it was found that conditioned medium from Dex-treated chondrocytes was able to stimulate migration of RAW264.7 cells as well as enhance formation of TRAP-positively stained RAW264.7 cells. In particular, inhibition of these promoting effects of the conditioned medium by a neutralising antibody for SDF-1 was also observed.

Discussion and Conclusion: These findings suggest that Dex treatment can cause apoptosis of chondrocytes and elevated expression of chemokines by apoptotic chondrocytes, which may be responsible for enhanced osteoclast migration and formation, serving as a potential molecular link between chondrocyte apoptosis and osteoclast differentiation.

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