University of Alberta

Effects of macrophages and noggin suppression on the BMP-2-induced osteogenesis of human bone marrow mesenchymal stem cells

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Experimental Surgery

Department of Surgery

Spring 2011
Edmonton, Alberta

Examining Committee

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ABSTRACT

The osteogenic effects of bone morphogenetic protein-2 (BMP-2) on human mesenchymal stem cells (MSCs) are less profound than expected as compared with rodent cells, and supraphysiological dose of BMP-2 is required to achieve desired clinical outcome. The mechanism for this phenomenon is unclear. In this study, we examined the effects of macrophages and noggin suppression on the BMP-2-induced osteogenesis of human bone marrow MSCs *in vitro*.

Our data show that macrophage conditioned medium significantly decreased the migration capacity, metabolic activity and BMP-2-induced osteogenesis of MSCs. In addition, knocking down noggin by small interfering RNA (siRNA) also significantly decreased BMP-2-induced osteogenesis and proliferation of MSCs.

In summary, our studies demonstrated that macrophages and knocking down the expression of noggin decreased BMP-2-induced osteogenesis of human MSCs *in vitro*. In the future, manipulation on macrophage activation and noggin expression may allow us to achieve higher BMP-2-induced osteogenesis that leads to better bone healing.

ACKNOWLEDGEMENT

First and foremost, I would like to thank my supervisor Dr. Hongxing Jiang for his kindness, enthusiasm and perseverance during my graduate training. Under his excellent guidance, I achieved a clearer concept on how to do research. His expertise and in-depth insight in both clinical problems and basic science research have brought me keen interest and inspiration on what I have been studying. Besides, I truly appreciate precious advice and support from my co-supervisors Dr. Hasan Uludag and Dr. Zhixiang Wang. Their sincere help allowed me to conduct my thesis research smoothly and efficiently.

Additionally, I would thank Dr. Yuling Fu for her inspiring suggestion and encouragement. I am thankful to Dr. Tom Churchill and Dr. Gina Rayat for their kind advice. I wish to thank all staff in the Department of Surgery for their care and support during my graduate study. I would like to thank all staff and students in Dr. Hasan Uludag's and Dr. Zhixiang Wang's labs for their kindly help.

I would like to extend my sincere gratitude to Dr. Hong Zhou who was my mentor for several years and encouraged me a lot down the road. Special thanks are owed to my family. Their love is the essential power of my advance.

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LIST OF ABBREVIATIONS

ACVR	Activin Receptor
ALP	Alkaline Phosphatase
APC	Adenomatous Polyposis Coli
BAMBI	BMP and Activin Membrane-bound Inhibitor
Bmper	BMP-binding Endothelial Cell Precursor-derived Regulator
BMPs	Bone Morphogenetic Proteins
BMPR	BMP Receptor
BSA	Bovine Serum Albumin
CEBPα	CAAT/Enhancer-Binding Protein α
CK1	Casein Kinase 1
CM	Conditioned Medium
Co-Smads	Common Partner Smads
DKK	Dickkopf Protein
DLX	Distal-Less Homeobox Transcription Factor
Dvl	Dishevelled Protein
dsRNA	Double-Stranded RNA
ECM	Extracellular Matrix
ESCs	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FGFs	Fibroblast Growth Factors
FHL	Four and a Half LIM-only Protein
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase Gene

GSK3-β	Glycogen Synthase Kinase 3-β
IBSP	Integrin-Binding Sialoprotein
IL-1β	Interleukin-1β
ISCT	International Society for Cellular Therapy
LRPs	Lipoprotein Related Proteins
MGM	MSCs Growth Medium
MSCs	Mesenchymal Stem Cells
OC	Osteocalcin
PBS	Phosphate Buffered Saline
PDGFs	Platelet-Derived Growth Factors
PMA	Phorbol-12-Myristate-13-Acetate
PPARγ	Peroxisome Proliferators-Activated Receptor γ
P-Smads	Phosphorylated Smads
RANKL	Receptor Activator of Nuclear Factor κB Ligand
rhBMP-2/7	Recombinant Human Bone Morphogenetic Protein-2/7
RISC	RNA-Induced Silencing Complex
RNase	Ribonuclease
RNAi	RNA Interference
R-Smads	Receptor-regulated Smads
RUNX2	Runt Homology Domain Transcription Factor 2
siRNAs	Small Interfering RNAs
TAZ	Transcription Coactivator with PDZ-binding Domain
TCF/LEF	T-Cell Factor/Lymphoid Enhancer Factor

TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
Tsg	Twisted Gastrulation

CHAPTER 1

GENERAL INTRODUCTION

Large bone injury, lesions and impaired healing may lead to bone non-union or loss of function. Bone grafts, which could provide mechanical and osteoinductive support for bone formation, are usually used to aid spinal fusion, fracture healing and reconstruction of bone defects resulting from trauma, bone disorder, or abnormal skeletal development [1] and therefore bone grafting is one of the most common procedures in orthopaedic surgery. Currently the "gold standard" solution for bone repair is autologous bone grafting in which patient's own bone is harvested from one part and transplanted to another part of the same patient to improve healing and functionality [2,3]. Autologous bone grafts possess excellent osteoconductivity, osteoinductivity, and osteogenic capability due to abundant growth factors, numerous osteogenic cells and no immunogenicity. However, autologous bone grafting is restricted by the limited volume of donor tissue and morbidity at the donor sites [3]. Another conventional procedure is allogenic bone grafting, in which bone grafts from patients (or cadavers) are transplanted to the injured sites of different patients. Although allogenic bone grafts show considerable osteoconductivity, they are far from ideal in clinical practice due to lack of osteogenic potential, poor osteoinductivity, less revascularization, remarkable immune rejection and risks of disease transmission [4,5]. Another conventional bone grafting is xenografting, in which bone grafts are harvested from a species other than human (e.g. porcine, bovine) and transplanted to the injured sites of human patients. Xenografting bone grafts also

show remarkable disadvantages, such as eliciting strong immunorejection [6] and transmitting disease [7,8]. Biomaterials, including metals, synthetic polymers and ceramics, are alternatives for autografts, allografts and xenografts, but they also show major drawbacks, such as no osteoinductivity and remarkable immune incompatibility.

Compared with the aforementioned conventional bone grafts, the bone grafts engineered with autologous stem cells, bioactive factors and scaffolds, attract more and more interest. In modern bone tissue engineering, extensive studies have been done on bioactive factors, postnatal stem cells and biomaterial-based scaffolds to enhance osteogenesis. A large number of bioactive factors, such as bone morphogenetic proteins (BMPs), Wnt signaling proteins, transforming growth factor-β proteins (TGF-βs), are currently being investigated for their osteoinductive capability [9]. Mesenchymal stem cells (MSCs) could be easily isolated from adults and induced to differentiate into osteoblasts, thus being an ideal cell source for bone tissue engineering. Biomaterial-based scaffolds can serve as a vehicle to control the release of bioactive factors and/or support proliferation, differentiation and transplantation of stem cells. Engineered bone grafts hold a great potential for facilitating bone regeneration due to the unparalleled osteogenesis, osteoinductivity and osteoconductivity. Rational design and fabrication of autologous bone grafts will remarkably benefit patients with bone fracture or bone disorders.

1.1 BONE BIOLOGY

1.1.1 Bone structure and physiology

Bone is one of the most important parts of locomotive system of human body. It mainly consists of calcified bone matrix, proteoglycans, calcium phosphate, and various cells [10]. Based on the structural properties, bone could be classified into trabecular bone and compact bone, which locate at different sites and play distinct roles. Bone provides mechanical support for muscles attachment and protects internal organs and the nerve system from injury. It also functions as a primary reservoir of calcium for the body and accommodates postnatal hematopoietic and mesenchymal stem cells [2].

There are mainly three kinds of cells involved in bone biology: osteoblasts, osteocytes, and osteoclasts. Osteoblasts, the bone-forming cells, originate from the mesenchymal stem cells residing in the bone marrow. The mature osteoblasts can continuously secrete type I collagen, proteoglycans, and glycoproteins to form extracellular matrix [11]. The extracellular matrix is then gradually mineralized to form bone matrix in the presence of alkaline phosphatase (ALP). Some of osteoblasts are buried in the mineralized extracellular matrix, becoming osteocytes. Unlike the fibroblast-shape osteoblasts, osteocytes are flat and almond-shape cells with reduced rough endoplasmic reticulum, Golgi complex and condensed nuclear chromatin. Osteocytes are actively involved in bone turnover, ion exchange, as well as functional adaptation of bone [12]. Osteocytes reside in the cavity (also called Lacunae) formed by the mineralized bone matrix,

with one osteocyte in each lacunae [13]; however, they remain in contact with each other through gap junction-coupled cell processes [12]. Osteoclasts, the bone-resorbing cells, are differentiated from the progenitors of hematopoietic lineage and osteoclast differentiation is regulated by osteoblasts [11,14]. Mature osteoclasts are large and branched motile cells, and usually adhere to bone matrix via a ruffled border. Osteoclasts contain acidic hydrolytic enzymes which dissolve calcium phosphate through acidification [13]. In normal postnatal life, bone mass stays in a dynamic equilibrium, which is maintained by the osteoblast-mediated bone formation and the osteoclast-mediated bone resorption [11]. This process is called remodeling, which is a dynamic change of bone to meet its mechanical and physiological requirements and to heal micro-injuries that occur from time to time. This process is constantly regulated by various hormones, growth factors, cytokines as well as other extracellular matrix molecules. Imbalance between bone formation and resorption will lead to bone structural disorders. For instance, bone resorption exceeding bone formation results in osteoporosis [15]; in contrast, bone formation exceeding bone resorption leads to osteopetrosis [16]. The aforementioned cells coordinately regulate survival, development and differentiation one another. For instance, osteoblasts could trigger differentiation and development of osteoclasts [14], whereas osteoclasts are capable to recruit human MSCs to the sites of bone remodeling, which then differentiate into osteoblasts to form new bone matrix [17].

1.1.2 Bone formation

There are two kinds of skeleton formation in mammal embryonic development, namely endochondral ossification and intramembranous bone formation. In the process of endochondral ossification, the cartilage anlagen is initially formed [18]. Chondrocytes in the central area of cartilage anlagen start off by turning into hypertrophic chondrocytes, and they induce angiogenesis by secreting angiogenic factors and then secrete collagen X extensively [19]. This process is called cartilage condensation. Osteoblasts, osteoclasts and hematopoietic cells migrate into the condensation area through blood vessels and form the ossification centers. Hypertrophic chondrocytes in these ossification centers are gradually replaced by osteoblasts. Finally trabecular bone and bone marrow formed in this region [18-20]. Endochondral bone formation starts at the early developmental stage and ceases after maturation. A typical endochondral bone formation could be observed in the growth plate of long bone. In the process of intramembranous bone formation, MSCs directly differentiate into osteoprogenitor cells or osteoblasts, which then secrete extracellular matrix as the foundation for later calcium deposition. The initial extracellular matrix is then mineralized into bone matrix, facilitated by ALP. In this process, some of the osteoblasts are embedded in the bone matrix and further differentiate into osteocytes [19]. Craniofacial skeleton and the clavicle are typically formed by means of intramembranous bone formation. The process of intramembranous bone formation continues in postnatal life of mammals.

1.1.3 Fracture healing after injury

Bone fracture causes the damage of blood vessels and discontinuity of mechanical structures of skeleton, and meanwhile it triggers complicated cell signaling networks to repair the lesions and restore bone function. Three biological stages in fracture healing are involved, namely inflammation, repair, and remodeling, in temporally sequential consequence [2]. Immediately after a bone fracture, bleeding at the injured sites forms haematoma (blood clots), and the vasoactive mediators, growth factors as well as inflammatory cytokines are released from the haematoma. Macrophages and other immune response cells are recruited to remove necrotic tissues on the site. MSCs are then attracted to this area by these bioactive factors, and differentiate into osteoprogenitor cells or osteoblasts. Extracellular matrix is secreted by mature osteoblasts and ossified to form immature woven bone. The osteoblasts, extracellular matrix, cartilage, new vessels and woven bone form fracture callus to fill up the gap of bone fragments. At the same time, osteoblasts and healing process will induce migration, differentiation and maturation of osteoclast progenitors [14]. Osteoblasts and osteoclasts work synergistically to remodel the newly formed callus under the regulation of different cell signaling pathways and the Wolff Law (bone is built up where mechanical load is aligned and absorbed where weight-bearing is not applied) until the function is completely restored [2].

1.2 SIGNALING PATHWAYS IN BONE DEVELOPMENT

A number of signaling pathways drive the bone formation and remodeling, including but not limited to BMP signaling, Wnt signaling, notch signaling and hedgehog signaling [21]. Among these pathways, BMP and Wnt signaling pathways are two key pathways in the regulation of osteogenesis.

1.2.1 BMP signaling

The osteoinductive activity of BMPs was firstly discovered in the 1965 [22] and since then its biological function on bone induction and metabolism has been extensively investigated. BMPs belong to the TGF-β superfamily and approximate 20 BMPs have been identified. BMP-2, 4, 6, 7, 9 have been reported to be able to induce osteogenic differentiation of mesenchymal progenitor cells [23,24]. Different types of BMPs induce or improve osteogenesis at different stages of osteogenic differentiation through complicated mechanisms in a temporal fashion. For example, BMP-2, 6, 9 were reported to engage in the transition of pluripotent MSCs towards osteoprogenitors; BMP-2, 4, 7, 9 involved in the differentiation process from osteoprogenitors to osteoblasts; and most BMPs promoted the maturation and terminal differentiation of osteoblasts [25]. Although BMP-2 and BMP-4 were not directly associated with the early stage of skeletal development [26], fracture healing in BMP-2 knockout mice failed, indicating that BMP-2 played an essential role in the initiation of bone fracture healing and bone regeneration in postnatal life [27]. During early osteogenic differentiation, BMP-7 promoted osteogenesis by up-regulating expression of

osteogenic marker genes and inhibiting cell cycle progression and cell proliferation [28]. Nonsense mutation of BMP-5 in mice impaired long bone width [29,30]. Constitutively active mutation of BMP type I receptor was reported to be associated with inherited fibrodysplasia ossificans progressiva [31]. Over-expression of constitutively active BMP type I receptor induced ectopic endochondral bone formation and joint fusion, whereas inhibition of BMP type I receptor resulted in reduced ectopic ossification and functional impairment [32].

Canonical BMP signaling pathway is shown in Figure 1-1. BMPs exert functions by binding to type I and type II receptors at the cell surface, which are all single-transmembrane serine/threonine kinases [28]. Type I receptors include activin receptor (ACVR) type IA and BMP receptor (BMPR) type IA, while type II receptors include ACVR IIA and BMPR II. Both type I and type II receptors consist of a signal peptide, an extracellular ligand-binding domain, a single transmembrane domain and an intracellular serine/threonine kinase domain. They are classified by the molecular weight and the type I receptor-specific glycine/cysteine-rich domain [33]. Upon binding, BMPs activate the type I receptor and subsequently phosphorylate receptor-regulated Smads (R-Smads, Smad 1, 5 and 8), allowing phosphorylated R-Smads (P-Smads, P-Smad 1, 5 and 8) to form a complex with the common partner Smad (Co-Smad, Smad 4). The Smads complex translocates into the nucleus and regulates transcription of the target genes directly or indirectly by interacting with various transcription factors, such as runt homology domain transcription factor 2 (RUNX2), the receptor activator

of nuclear factor κB ligand (RANKL), and osterix, etc [34]. RUNX2 has been well characterized as a crucial transcriptional factor, which interacts with P-Smads, to regulate BMPs-induced osteogenic differentiation of stem cells [35-38]. The HTY (aa426-428) region at the C-terminal domain of RUNX2 is the key region for governing the interaction between RUNX2 and P-Smads, leading to the downstream osteoblasteogenesis [39]. By analyzing the microRNA profiling in BMP-2-induced osteogenesis and selectively down-regulating osteogenesis-promoting genes, Li Z et al showed that BMP-2 regulated osteogenic differentiation through Smad5 and RUNX2 [40]. Smad proteins could also activate RUNX2 indirectly through homeodomain proteins distal-less homeobox transcription factor (DLX)3 and DLX5 [41] or the transcription coactivator with PDZ-binding domain (TAZ) [42] to regulate downstream osteogenic differentiation. Osterix is one of the downstream transcription factors of RUNX2. It was found that osterix deficiency resulted in failure of osteogenic differentiation and bone formation [43]. The target genes regulated by RUNX2 also include osteocalcin, bone sialoprotein, ALP, and type I collagen, etc [44].

The osteoinductive activity of BMPs is precisely controlled by both positive and negative feedback mechanisms in order to ensure adequate amount of bone formation at the proper site and at the right time. Endogenous BMPs are induced during osteogenic differentiation of pluripotent mesenchymal stromal cells [45] and osteogenic gene expression is up-regulated by BMPs stimulation [46].

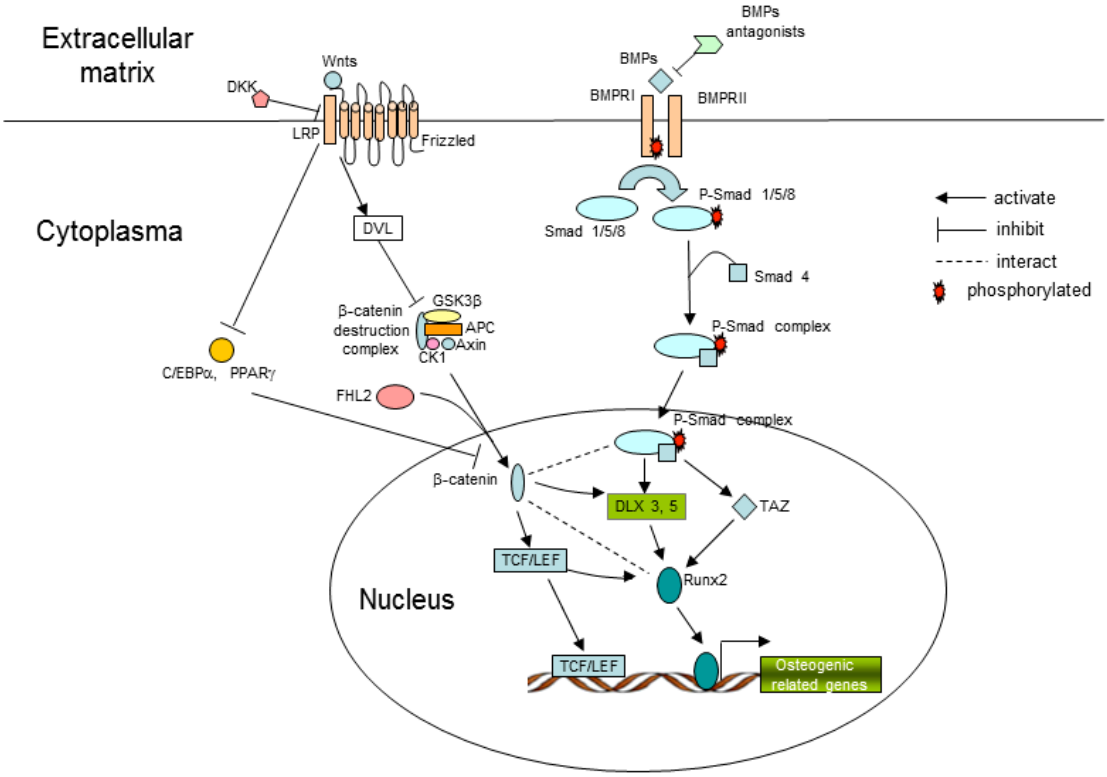

Figure 1-1. BMP and Wnt signaling pathways. BMPs transduce signals via type I and II BMP receptors and their downstream Smad 1, 5 and 8 proteins. Phosphorylated Smad 1, 5 and 8 form a complex with Smad 4 and are transolated into the nucleus where they interact with transcription factors to regulate the transcription of osteogenic genes. Wnts bind to Frizzled/LRP receptor complex and transduce a signal to DVL, which inhibits the degradation of β-catenin. β-catenin accumulates in the cytoplasm and nucleus, and interacts with TCF/LEF to control the transcription of genes. Abbreviations: APC, adenomatous polyposis coli; BMPs, bone morphogenetic proteins; C/EBPα, CCAAT/enhancer binding protein α; CK1, casein kinase 1; DKK, Dickkopf protein; DLX, distal-less homeobox transcription factor; DVL, Dishevelled Protein; FHL, four and a half LIM-only protein; GSK3β, glycogen synthase kinase 3β; LRP: lipoprotein receptor-related protein; PPARγ, peroxisome proliferator-activated receptor γ; P-Smad, phosphorylated Smad; RUNX2, runt homology domain transcription factor 2; TAZ, the transcription coactivator with PDZ-binding domain; TCF/LEF, T-cell factor/lymphoid enhancer factor; Wnts, wnt proteins.

Extracellular antagonists prevent BMPs from binding to their receptors and impair BMP signaling for osteogenesis [47]. Many extracellular antagonists of BMPs are well characterized, including noggin, chordin, follistatin, cerberus,

inhibin, gremlin, and twisted gastrulation (Tsg) [48,49]. Studies have shown noggin plays an important role in bone formation [50,51]. Noggin blocks BMP signaling by binding to BMPs and preventing BMPs from binding to BMP receptors. The "finger" structure and the C-terminal half of noggin bind to BMP type II receptor (ActRII), and the N-terminal half of noggin binds to BMP type I receptor (BMPRIA) (Figure 1-2) [52]. Detailed roles of noggin in osteogenic differentiation will be discussed in Chapter 3.

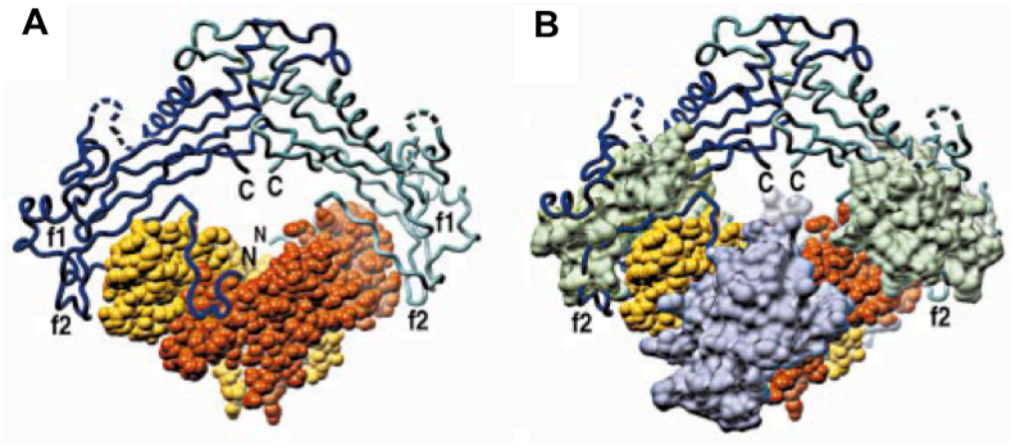

Figure 1-2. Structural basis of noggin blocking BMP-7-ActRII-BMPRIA interaction. Noggin-BMP-7 complex (A) is superimposed on a BMP-7-ActRII-BMPRIA complex model (B). The hydrophobic patches on the BMP-7 (red and yellow space-filling) are masked by noggin (blue and green coil), which prevents BMP-7 from binding to BMPRIA (blue space-filling) and ActRII (Green space-filling). Adapted from: Groppe J, et al. (2002) Structural basis of BMP signaling inhibition by the cystine knot protein Noggin. Nature 420:636-642 [52].

BMP signaling-mediated osteoinduction has shown a significant discrepancy among different species. Human MSCs show a poor responsiveness towards BMPs for their committed osteogenesis *in vitro* in comparison with rodent MSCs [53,54]. The high dose of recombinant BMP-2 (~1.5 mg/ml) is usually

required to treat delayed-union or non-union bone fractures in clinical setting, whereas a dose at the μg/ml level induces a large amount of ectopic bone formation in mice [55,56]. The reasons for this species-specific effectiveness of BMPs are largely unknown.

1.2.2 Wnt signaling

In addition to BMP signaling pathway, Wnt signaling pathway is also crucial for osteogenesis regulation. The Wnts are lipid-modified secreted proteins that function in various developmental and physiological processes. Wnt signaling is regarded as the switch for osteogenic/adipogenic differentiation. Activation of Wnt signaling improves osteogenesis and inhibited adipogenesis, whereas inactivation of Wnt signaling leads to the opposite effects [57-59]. A constant low level of Wnt signaling is crucial for proliferation of MSCs and a high level of Wnt signal leads to initiation of osteogenesis [60].

Canonical Wnt signaling pathway is shown in Figure 1-1. In the absence of active Wnts, intracellular β-catenin is entrapped by a β-catenin destruction complex composed of glycogen synthase kinase 3β (GSK3β), Axin, adenomatous polyposis coli (APC), and casein kinase 1 (CK1) [61]. In the complex, β-catenin is phosphorylated, ubiquitinated and degraded by proteasomes [62]. In the presence of Wnts, Wnts bind to the frizzled receptors and low-density lipoprotein receptor-related proteins (LRPs) and then activate the dishevelled protein (Dvl), which subsequently inhibits the destruction complex

formation and release β-catenin. β-catenin is then translocated into the nucleus and binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) to regulate target gene transcription [61]. Wnts increase bone formation by enhancing osteogenic differentiation [63], inhibiting adipogenic differentiation [64] as well as inhibiting osteoblast/osteocyte apoptosis [57]. Wnt11 facilitates osteoblast maturation and mineralization through the β-catenin and R-spondin 2 pathway [65]. Wnt10b increases osteogenesis by activating RUNX2, DLX5, osterix and suppressing CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferators-activated receptor γ (PPARγ) [66,67]. Four and a half LIM-only protein 2 (FHL2) mediates dexamethasone-induced osteogenic differentiation of MSCs through the Wnt/β-catenin pathway [68].

Many studies have indicated the crosstalk between BMP signaling and Wnt signaling [69-71]. During osteogenic differentiation, BMPs induce Wnts expression and activate Wnt/LRP5/β-catenin signaling pathway which regulates ALP activity in an autocrine/paracrine manner [72]. Tang et al. have found that β-catenin suppression leads to decreased ALP activity, reduced expression of osteogenic markers and mineralization *in vitro* and impaired ectopic bone formation *in vivo* [69]. Canonical Wnt/β-catenin signaling is usually required in BMP-9-induced osteogenic differentiation of mesenchymal progenitors [69]. A Wnt autocrine loop is involved in mediating BMP-2-induced ALP expression and extracellular matrix mineralization [72]. During osteogenic differentiation of C2C12 mesenchymal cells, BMP-2 down-regulates microRNAs which could

target to members of Wnt/β-catenin signaling pathway [40], indicating Wnt signaling pathway is regulated by BMP-2 signaling.

1.3 BONE TISSUE ENGINEERING

1.3.1 Bioactive molecules

Many kinds of molecules affect proliferation and differentiation of osteogenic progenitor cells, such as growth factors, chemical molecules, hormones, as well as cytokines. Since the first discovery of BMPs in bone matrix in 1965 [22], many growth factors associated with osteogenesis have been identified, including TGF-βs, Wnts, fibroblast growth factors (FGFs), platelet-derived growth factors (PDGFs), etc.

BMPs are the most extensively investigated factors in bone research. BMP-2 and BMP-7 have been approved by FDA for treating critical-size bone fracture and spinal fusion clinically. However, human clinical trials have shown that BMPs are much less effective than expected [53]. The reasons for low responsiveness of human MSCs towards BMPs are still largely unknown. Recently, many studies have focused on how to improve the osteoinductive capacity of BMPs. One of the most promising strategies is to create a "cocktail" combination of BMPs with small molecular chemicals or other bioactive molecules. It is known that BMP-2 are heparin-binding proteins and its bioactivity for osteogenic differentiation can be improved by heparin sulfate [73,74], synthetic heparin-like dextran derivatives [75] and 2-N, 6-O-sulfated chitosan [76]. Some other chemical molecules, including N-

methylpyrrolidone [77], pentoxifylline [78,79], rolipram [80], 3',5'-cyclic adenosine monophosphate [81], have also been used to enhance BMP signaling in osteogenesis. The incentives of using simple chemicals include: 1) cost-effectiveness, simple chemicals are much cheaper than large molecular proteins; 2) defined characteristics, simple chemicals are less likely to cause other side effects when applied *in vivo*. So far there has been no solid conclusion for single small molecular chemicals that significantly enhanced BMP-mediated bone formation. Another promising strategy is genetic modification. The BMP-2/7 heterodimers significantly increased ALP activity and up-regulated osteocalcin expression, with a mild induction of noggin expression comparing with individual BMP-2 or BMP-7 homodimers [82]. A lot of effort has been focused on increasing the life of BMPs in the application sites. It was reported that the concentration of BMP-2 in the culture medium rapidly decreased to below 50% of the original concentration after 1 hour and was almost undetectable after 10 hours due to degradation [74]. The mechanisms for this phenomenon are not yet clear to date although proteases in the culture might cause degradation of BMPs [83]. It was also assumed that Bmper (BMP-binding endothelial cell precursor-derived regulator), noggin and gremlin could trigger endocytosis of BMP-4, followed by degradation by lysosome [84]. Heparin may effectively protect BMP-2 from degradation and significantly retain BMP-2 activity for a prolonged period [73,74]. Coated with bovine serum albumin (BSA) nanoparticles, BMP-2 could be retained longer on applied sites [85].

1.3.2 Mesenchymal stem cells

Stem cell research has promoted its development in both basic science research and preclinical trials over past decades. Engraftment of MSCs has been investigated in experimental autoimmune encephalomyelitis [86], renal injury [87], heart infarction [88], neural injury [89], and steroid-resistant acute graft-versus-host diseases [90,91]. It has been reported that implantation of MSCs improved formation of bone mass, accelerated bone healing and increased osteogenesis in osteogenesis imperfecta [92,93]. Implantation of culture-expanded MSCs into the distracted callus accelerated bone healing and shortened the treatment period in distraction osteogenesis [94].

MSCs are anticipated to be the excellent cellular component for bone tissue engineering in the future in that MSCs 1) can be easily isolated, 2) possess great expansion potential *in vitro*, 3) have less ethical controversy than that of using embryonic stem cells (ESCs) in clinical therapy, 4) elicit no or mild host immune response cross the individual transplantation, and 5) accelerate the repair process of damaged bone or even adjacent tissues [95,96]. Bone marrow is regarded to be the most abundant source of MSCs. In orthopaedic surgery, bone marrow could be harvested from the injured and opened medullar canal of bone. After purification from bone marrow, MSCs could be manipulated *in vitro* in various ways. One common method is to expand MSCs in the cell culture system in early passages, seed them on a scaffold, induce osteogenic differentiation and calcium deposition by bioactive molecules and chemicals, and then implant the

tissue-engineered graft to the patient. Another approach is to expand MSCs in cell culture system, induce the osteogenic differentiation and proliferation to a magnificent numbers *in vitro*, and reconstitute scaffolds with mature osteoblasts to form a ready-to-use tissue-engineered composite graft for clinical application. It has been shown that the tissue-engineered bone graft, which was made of allogenic MSCs and a coral scaffold, successfully healed the bone defects with a critical size in sheep [97]. In the first clinical report for MSCs-based bone tissue engineering, all three patients showed abundant callus formation around the implants and achieved excellent integration at the interfaces between the implants and host bones [98].

1.3.3 Scaffolds

In bone tissue engineering, scaffolds not only provide mechanical and functional support for cells and bioactive molecules, but also act as a reservoir, providing appropriate microenvironment for mineralization of newly formed bone matrix. Ideal scaffolds for bone tissue engineering should have at least the following characteristics: 1) have no toxicity, 2) have appropriate structure and properties for cell attachment, proliferation and differentiation, 3) could be eliminated after completion of bone healing process. Scaffolds could be classified to be organic and inorganic materials [99]. A variety of scaffolds were used as a carrier for MSC growth, differentiation, and transplantation [100]. Considering the critical roles of extracellular matrix (ECM) in bone morphogenesis, a variety of artificial ECM structural mimics have been developed for bone tissue engineering [101]. Although

this kind of scaffold could not provide MSCs with comprehensive bioactive molecules temporally and spatially as the natural ECM for bone regeneration, it holds a great potential along with rapid progress in the knowledge on cell signaling, bioactive molecules and biomaterials.

1.4 PERSPECTIVES

In despite of showing great potential for aiding bone healing, tissue-engineered bone still has a long way to go prior to its wide clinical application. Several issues should be considered to further improve the osteogenic outcomes and the safety. The first concern is the standard for postnatal MSCs manipulation. Different laboratories have established their own methods to isolate and expand MSCs, which may generate slight to significant differences in tissue-engineered bone grafts. The International Society for Cellular Therapy (ISCT) has proposed several standards for defining and characterizing MSCs [102,103]. However, standardization in operation of MSCs is still needed. The second concern is how to expand MSCs and drive its osteogenic differentiation under 3-dimentional (3D) culture conditions. Although several 3D culture systems have been explored [104-106], challenges still exist to culture and differentiate MSCs in 3D conditions without reducing proliferation and differentiation capacity. The third one is the hidden risks brought by the *in vitro* culture system when we eventually bring tissue-engineered bone graft into clinical application, for instance, fetal bovine serum (FBS) might transmit the bovine spongiform encephalopathy and lead to immunological reactions. Alternative approaches have been tested to

solve this problem. Recently, the feasibility and efficacy of cultivating human MSCs in serum-free medium for bone tissue engineering were evaluated [107]. Also, human-derived alternatives for FBS were studied in the expansion of MSCs [108-110]. Interestingly, these studies indicated that cultured in these alternative supplements, MSCs showed similar surface marker profile, comparable proliferation rate and differentiation capacity, compared with MSCs cultured in FBS-supplemented medium. The fourth is the cell survival after implantation of engineered bone graft. Although several promising strategies has been tested on this issue [1,111,112], much more studies are needed to tackle this problem.

Although BMPs are potent for enhancing fracture healing and bone regeneration, there are still unsolved issues requiring extensive research. The first one is how to boost the osteogenic potential of BMPs through extending its retention time of BMPs both *in vivo* and *in vitro*. The underlying mechanism of BMP-mediated osteogenic dynamics and its interaction with the microenvironments where BMPs naturally occur should be investigated. The second one is to understand the reason(s) of relatively poor responsiveness of human tissues and cells towards BMPs. The poor responsiveness has led to requirement of a supraphysiological dose of BMP-2 for bone tissue engineering and clinical therapy, increasing health care burden for the treatment as well as side effects associated with large dose.

In summary, bone healing is a complex process that is tightly regulated by the immune system. Macrophages are one of the most prominent immune cells infiltrating the injured site after bone fracture. In normal bone fracture healing, macrophages are present in large numbers in the injured site at the early stage but become less in the bone formation area at the late stage [113]. While in delayed union and non-union fractures, CD11b-positive macrophages are consistently distributed in the connective tissue stroma with perivascular enrichment as long as 4-25 months after the bone fracture [114]. These observations indicate that macrophages might be involved in regulating bone fracture healing. Although BMPs are very potent in promoting the bone healing process, they are precisely regulated by various factors. Among them, noggin has been extensively investigated for its role in bone formation. However, the data regarding the effect of noggin on BMP-induced osteogenesis of MSCs are controversial. Most studies performed in rodent cells/models indicate that noggin is a negative regulator of BMP-2-induced osteogenesis; however, one study conducted with human MSCs in culture show that the addition of noggin induces osteogenesis *in vitro*. Thus we hypothesize that macrophages and noggin might play roles during the osteogenesis of human MSCs in the presence of BMP-2, and aim to examine the effects of macrophages and noggin suppression on the BMP-2-induced osteogenesis of human MSCs *in vitro*.

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CHAPTER 2

MACROPHAGES INHIBIT MIGRATION, METABOLIC ACTIVITY AND OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS *IN VITRO*

2.1 INTRODUCTION

There are 6.2 million cases of bone fractures annually in the United States alone, and roughly one in ten patients will develop a delayed union or non-union [1]. After a bone fracture, mesenchymal stem cells (MSCs), putative progenitors of osteoblasts residing in the bone marrow, migrate to the injured sites in response to inflammatory cytokine signals. It is then followed by proliferation and osteogenic differentiation of MSCs to produce bone matrix and repair the fractured bone. Recombinant human bone morphogenetic protein-2 (rhBMP-2) is widely used clinically to promote bone healing and spinal fusion by stimulating MSCs and osteoblasts [2]. The osteogenic effects of BMPs on MSCs are less profound than expected in the clinical setting and the mechanism for this phenomenon is unclear.

The immune system is tightly involved in the soft tissue repair process. Macrophages that accumulate at the injured sites at the very early stage were found to be alternatively activated and functioned as "repair macrophages" in wound healing [3,4]. The roles of macrophages in soft tissue repair were attributed to debride necrotic tissue [5], to fight infection [4], and to modulate repair response via secreting cytokines [6]. Depletion of macrophages in mice impaired the soft

tissue repair process. In the murine cryoinjury model, infiltrating macrophage depletion by clodronate liposomes impaired wound healing and increased left ventricular remodelling after myocardial injury [5]. In addition, wound healing phase-specific depletion of murine macrophages by diphtheria toxin indicated that early stage macrophage depletion resulted in impaired vascularization, epithelialization and minimized scar formation; mid-stage macrophage depletion resulted in severe haemorrhage in wound tissue; while late stage macrophage depletion did not significantly affect the healing process [7]. However, little is known about macrophages' role in bone healing and their interaction with MSCs, specifically in the presence of BMP-2.

Accordingly, we designed this study to examine the effect of macrophages on MSCs during BMP-2-induced osteogenesis. Specifically using the conditioned medium of macrophages, we assessed the effects of macrophages on the migration, metabolic activities, and osteogenic differentiation of MSCs.

2.2 MATERIALS AND METHODS

2.2.1 Isolation and expansion of human MSCs

Bone marrow samples were obtained from patients who underwent orthopaedic surgery after receiving their informed consent. The study was approved by the Research Ethics Committee at the University of Alberta. Mononuclear cells from the bone marrow were isolated by centrifugation (400 g, 25 min) using Ficoll-Paque (GE Healthcare, Piscataway, NJ, USA) and then were seeded at a density

of 4×10^5 cells/cm^2 in MSC growth medium (MGM: high glucose DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml Glutamax (all from Invitrogen, Carlsbad, CA, USA) and 4 ng/ml FGF-2 (Millipore, Temecula, MA, USA)). After being incubated for 3 days, non-adherent cells were discarded. Adherent cells were washed twice with phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) and expanded in MGM. After 7 days, the cells were either further expanded for experiments or frozen in 1 ml aliquots in liquid nitrogen. For all experiments, cells were incubated at 37 ^oC in a humidified 5% CO_2 atmosphere.

2.2.2 Fluorescence activating cell sorting analysis

After detaching MSCs with 0.05% trypsin (w/v) and washing with PBS, we incubated MSCs with PE-labeled anti-CD13, anti-CD45, and anti-CD105 (Caltag Laboratories, Burlingame, CA, USA) for 30 min, separately. MSCs were then fixed with 1% formaldehyde, resuspended in PBS and analyzed with the FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

2.2.3 Multi-lineage differentiation of MSCs

Multi-lineage differentiation capacity of isolated MSCs was confirmed with commercial Human Mesenchymal Stem Cell Functional Identification Kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. To induce osteogenic differentiation, human MSCs were cultured in the osteogenic medium (OGM) consisting of DMEM supplemented with 10% FBS,

100 U/ml penicillin, 100 mg/ml streptomycin, 0.29 mg/ml L-glutamine (all from Invitrogen, Carlsbad, CA, USA), 10 nM dexamethasone, 10 mM β-glycerolphosphate, and 0.05 mM ascorbic acid-2-phosphate (all from Sigma, St. Louis, MO, USA). After 28 days, the calcium deposit from induced osteogenic cells was stained with Alizarine Red (Sigma, St. Louis, MO, USA). To examine adipogenic potential, MSCs were expanded until 80% confluence and then treated with Stempro adipogenic differentiation medium (Invitrogen, Carlsbad, CA, USA). After 21 days, Oil Red O (Sigma, St. Louis, MO, USA) was employed to detect the formation of lipid droplets. For chondrogenic differentiation, MSCs were cultured in Stempro chondrogenic differentiation medium (Invitrogen, Carlsbad, CA, USA) for 21 days and were then stained with Alcian Blue (Sigma, St. Louis, MO, USA). In parallel, MSCs cultured in MGM were stained by Alizarine Red, Oil Red O, or Alcian Blue, respectively, to serve as the negative control for multi-lineage differentiation. All media were changed every 3 days.

2.2.4 Preparation of macrophage/monocyte conditioned media

Because primary tissue macrophages cannot be easily expanded *in vitro*, macrophages are usually obtained from *in vitro* cultured primary monocytes. Macrophages induced from THP-1 monocyte cell line by phorbol-12-myristate-13-acetate (PMA) were widely used as an *in vitro* model to investigate the function of macrophages [8,9], as they exhibit similar cell morphology, cytoplasmic to nuclear ratio, differentiation-dependent cell surface markers, and

phagocytic capacity as compared to primary monocyte-derived macrophages [10]. In this study, we used PMA-induced macrophages from THP-1 cells. The human myelomonocytic THP-1 cell line (202-TIB; a gift from Dr. Dean Befus, University of Alberta, Edmonton, AB) was recovered in the basal medium (RPMI 1640 medium (American Type Culture Collection, Manassas, VA, USA) containing 10% FBS (Invitrogen, Carlsbad, CA, USA) and 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA)) and was then maintained in the basal medium in suspension at 0.5-1.0×10^6 cells/ml. The THP-1 cells were then induced by PMA into adherent cells with macrophage characteristics [11], which was achieved by incubating THP-1 cells in basal medium containing 50 ng/ml PMA (Sigma, St. Louis, MO, USA). After 72 hours, the differentiated cells were washed three times with PBS and cultured in fresh basal medium for additional 24 hours. The macrophage conditioned medium (supernatant) was then collected, filtered with 0.22 μm filters (Millipore, Temecula, MA, USA), and stored at -20 ^{o}C for study. Basal medium and monocyte CM were prepared in parallel.

2.2.5 *In vitro* phagocytic assay

PMA-induced macrophages were incubated with 1 μm blue-dyed microspheres (10 microspheres/cell, Polysciences, Warrington, PA, USA) at 37 ^{o}C for 4 hours. Parallel control group of the THP-1 monocytes were also allowed to phagocytise microspheres under the same condition as macrophages. Cells were visualized after the incubation and photos were recorded under a light microscope.

2.2.6 Cell migration assay

The effect of macrophage CM on migratory capacity of MSCs was determined by transwell plates (Corning, Corning, NY, USA) with 6.5-mm diameter and 8-μm pore filters (Figure 2-1). Briefly, 3×10^3 MSCs in 100 μl MGM were seeded in the upper chamber of the transwell system and were incubated at 37 ^{o}C for 3 hours. Study groups with basal medium alone, basal medium containing 20% monocyte CM, or basal medium containing 20% macrophage CM were then added separately to the lower chamber of each transwell system for testing. Fifteen hours later, the filters were fixed with 10% formalin for 5 min and then stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA) for 30 min. Stained filter were rinsed 3 times with tap water and then examined under a light microscope.

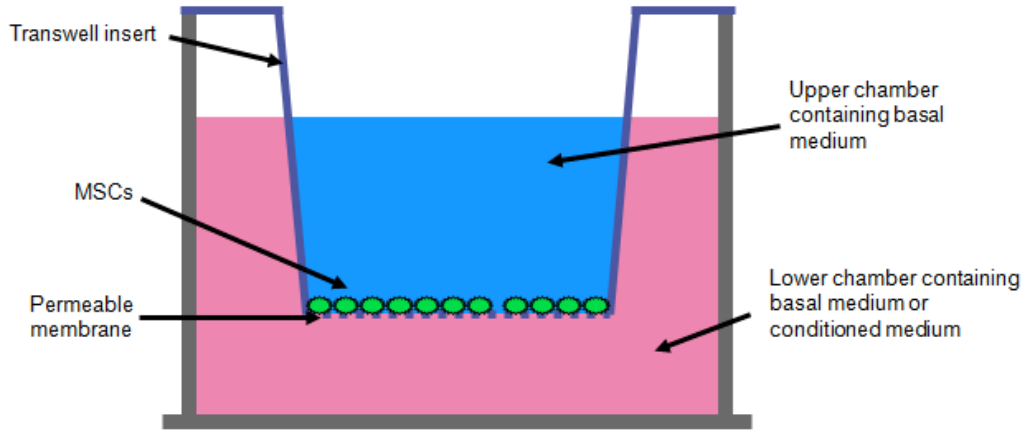

Figure 2-1. Diagrammatic representation of the transwell system. MSCs were seeded in the upper chamber containing basal medium in the upper chamber. Basal medium containing conditioned medium was added to the lower chamber. MSCs may migrate to the underside of permeable membrane in response to the soluble factors in the conditioned medium.

The number of migrated MSCs in 10 different view fields were counted and compared among groups. For the concentration-dependent assay, basal medium containing 0%, 20%, 40%, 60%, 80%, and 100% macrophage CM were added separately to each lower chamber of transwell system for testing in the same fashion as described above.

2.2.7 WST-8 assay for cell metabolic activity

MSCs were seeded in 48-well plates at a density of 5×10^3 cells per well in MGM one day before the assay. Basal medium and basal medium containing 20% monocyte CM or 20% macrophage CM were added into these wells, respectively; and the metabolism of MSCs was then assessed on the days 0, 3 and 7. A Cell Counting Kit-8 (Cedarlane, Burlington, ON, Canada) was used to count living cells by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, water soluble tetrazolium salt) assay. Briefly, 30 μl of the WST-8 solution was added to the 300 μl medium, and incubated for additional 3 hours. Absorbance was measured with a microplate reader (BioTek, Winooski, VT, USA) at 450 nm with a reference wavelength of 650 nm. Higher absorbance indicates more living cells. For CM concentration-dependent assay, MSCs were seeded in the same fashion as described above and treated with basal medium containing 0%, 20%, 40%, 60%, 80%, and 100% macrophage CM, respectively. Cell metabolism was assessed by WST-8 assay as described above on day 3 after treatment.

2.2.8 DNA content analysis

MSCs were seeded in 96-well plates at a density of 1×10^3 cells per well and cultured in MGM for 24 hours. Media were then replaced with basal medium, basal medium containing 20% monocyte CM, or basal medium containing 20% macrophage CM. At the indicated time points (on the days 0, 3 and 7 after treatment), cells were lysated and samples were collected. The DNA content was measured by using a CyQUANT Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA, USA) with the excitation light of 450 nm and emission wavelength of 530 nm. Higher fluorescence indicates more cells. Bacteriophage λDNA of known concentrations was used to create the standard curve to transform data. For CM concentration-dependent assay, MSCs were treated with basal medium containing 0%, 20%, 40%, 60%, 80%, and 100% macrophage CM, respectively. DNA content was measured as described above on day 3 after treatment.

2.2.9 RT-PCR and real-time quantitative PCR analysis

Total RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA, USA), purified by phenol/chloroform method and reverse transcribed to cDNA with the iScript cDNA Synthesis Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's protocol. Reverse transcription was performed in 20 μL of final volume with the following protocol: 25°C for 5 min followed by 42°C for 5 min, and 85°C for 5 min.

Expression levels of macrophage differentiation marker genes (CD36, CD11b, CD14) were examined by using RT-PCR and β-actin was used as the endogenous reference gene. The amplification protocol was one cycle of 94°C for 5 min, 35 cycles of 94°C for 30 sec, 62°C for 30 sec, 72°C for 1 min, followed by one cycle of 72°C for 5 min. PCR products were separated on a 1.5% agarose gel, stained with the 0.5 μg/ml ethidium bromide solution and visualized by using the ChemLite luminant image analysis system (Avegene, Taipei, TW, China).

Osteogenic differentiation of MSCs was examined by measuring the mRNA level of the following osteogenic marker genes with real-time quantitative PCR, alkaline phosphatase (ALP), integrin-binding sialoprotein (IBSP), runt-related transcription factor-2 (RUNX2), and osteocalcin (OC). Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as an endogenous reference gene to normalize target gene expression. Real-time quantitative PCR amplifications were conducted in quadruplicate with the iQ5 system (Bio-Rad, Mississauga, ON, Canada). The 25 μl reaction mixture contained 10 ng cDNA from reverse transcription of an individual sample, 200 nM of each primer, and 1× iQ SYBR Green supermix (Bio-Rad, Mississauga, ON, Canada). Real-time quantitative PCR was performed with the following protocols: one cycle of 95°C for 3 min, followed by 45 cycles of 95°C for 10 sec, 58°C for 20 sec, and 72°C for 10 sec. All primers (Invitrogen, Carlsbad, CA, USA) used in this study are listed in Table 2-1.

Table 2-1. Sequences of PCR primers used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CD36	GAATCCGACGTTAATCTGAAAGG	TTCCAGTTACTTGACTTCTGAAC
CD11b	GGCCAATGTGACCAGTGAGAAC	GAGGGCCAGGAGCAGCAGTC
CD14	AGCCTAGACCTGTCTGACAATC	CACCGACAGGGTCGAACGTG
β-actin	GCCCAGTCCTCTCCCAAGTC	GGCACGAAGGC TCATCATTC
GAPDH	GGACTCATGACCACAGTCCAT	CAGGGATGATGTTCTGGAGAG
ALP	ACTCCCACTTCATCTGGAACC	CCTGTTCAGCTCGTACTGCAT
IBSP	ACAGGGTTAGCTGCAATCCA	TGCCTTGTTCGTTTTCATCC
RUNX2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA
OC	GAAGCCCAGCGGTGCA	CACTACCTCGCTGCCCTCC

2.2.10 Alkaline phosphatase assay

After being treated with basal medium or basal medium containing 20% monocyte CM or macrophage CM for two days, MSCs were induced to undergo osteogenic differentiation by OGM containing 0.1 μg/ml BMP-2. After 14 days, ALP activity was quantitatively measured by a colorimetric assay (BioAssay systems, Hayward, CA, USA) according to the manufacturer's instructions. The cells in triplicate cultures were detached with lysis buffer containing 0.5% Triton, 50 mM Tris-HCl, and 5 mM $MgCl_2$ and collected individually. The lysate was then transferred to 96-well plates, incubated with ALP substrate at 37°C for 30 min and then the reaction was halted with the stop buffer. The p-nitrophenol product formed by enzymatic hydrolysis of p-nitrophenylphospate substrate was measured at 405 nm with the microplate reader. Protein concentration of ALP samples was measured with a DC Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions and bovine serum albumin (Bio-Rad, Mississauga, ON, Canada) was used to create a standard curve to transform data. ALP concentration was normalized by the total protein amount.

2.2.11 Alkaline phosphatase staining

On the day 14 of osteogenic induction, ALP staining was performed with a Fast Blue B Kit (Sigma, St. Louis, MO, USA). Briefly, cells were washed three times with PBS and fixed by citrate-acetone-formaldehyde fixative for 30 sec. After a brief rinse with deionized water, samples were stained by sodium nitrite/FBB alkaline solution in the dark for 15 min, and counterstained by neutral red solution for 2 min. Finally, samples were washed three times with tap water to remove the dissociative dye.

2.2.12 Calcium deposition assay

The cells and extracellular matrix secreted by the cells were lysated and demineralized by adding 600 μl of 0.5 N HCl to each well (12-well plates) and incubating at 4°C overnight. The supernatant containing calcium extracts was collected after centrifugation at 10,000 g for 10 min. The calcium concentrations were measured with a QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions.

2.2.13 Alizarin red staining

On the day 28 of osteogenic induction, cells and the extracellular matrix of all groups were incubated in 75% alcohol at 4°C for 1 hour, rinsed rapidly in distilled water, and stained by Alizarin red S solution (Sigma, St. Louis, MO, USA) for 30 sec to 5 min until orange-red color. After removing excess dyes, 20

dips acetone and 20 dips acetone-xylene were added separately, followed by clearing in xylene.

2.2.14 Statistical analysis

Data was presented as the mean with the standard deviation and analyzed by one-way ANOVA followed by Bonferroni post-hoc test. All tests were 2-sided with $p<0.05$ considered as the level of significance. All analyses were performed with PASW Statistics 18.0 (SPSS, Chicago, IL, USA).

2.3 RESULTS

2.3.1 Characterization of MSCs and PMA-induced macrophages

MSCs from human bone marrow adhered to plastic tissue culture dishes and displayed the spindle shape of typical MSCs' characteristics (Figure 2-1, A). The MSC surface markers CD105 and CD13 were presented in the cell cultures and the hematopoietic cell marker CD45 was absent (Figure 2-1, B). Established MSCs were able to undergo adipogenic, chondrogenic, and osteogenic differentiation under standard tissue culture-differentiating conditions *in vitro* (Figure 2-1, C, lower panel). These characteristics of established MSCs fulfill the three criteria for MSCs proposed by the International Society for Cellular Therapy and are also in accordance with previous observations [12-14].

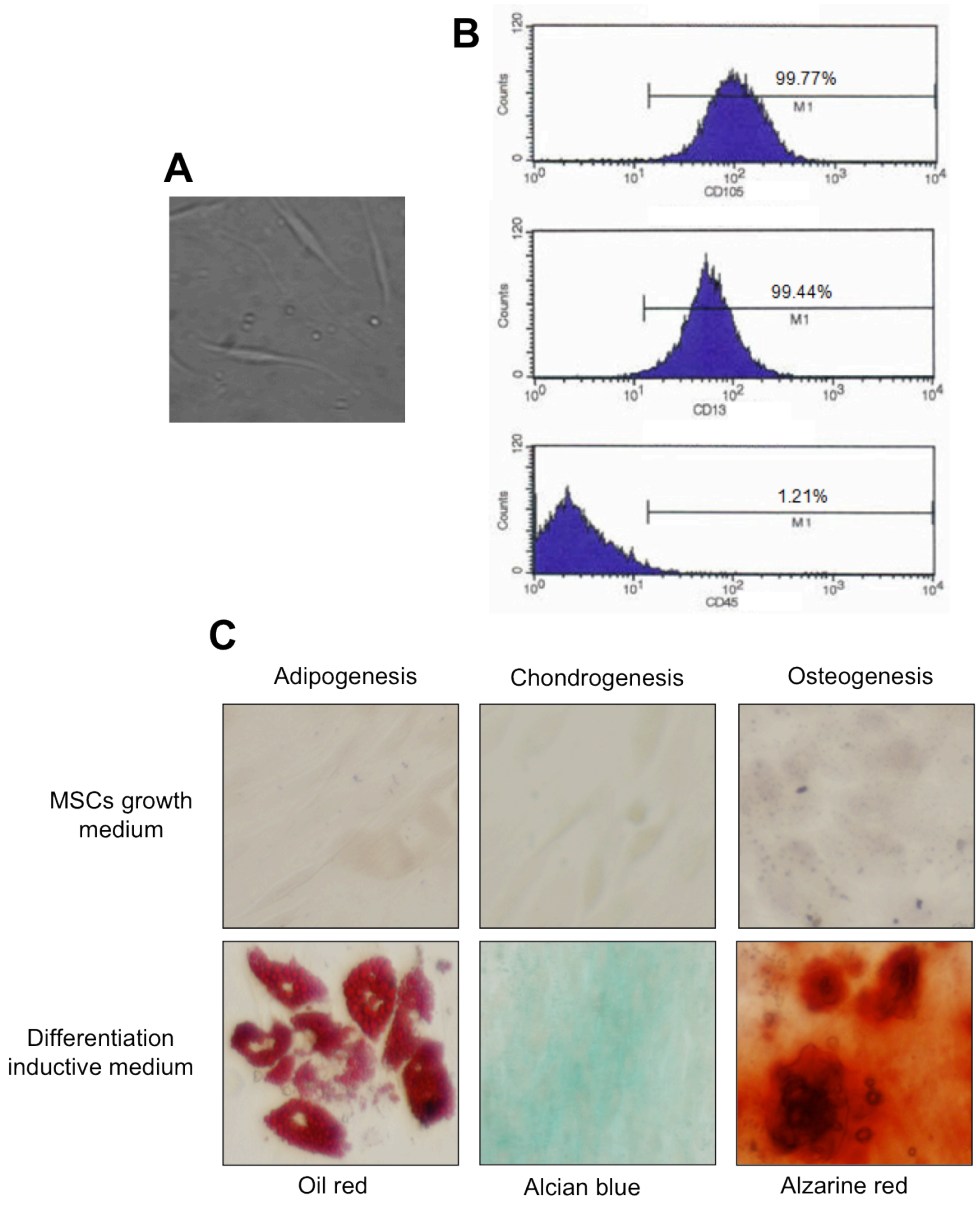

Figure 2-2. (A) Morphology of *in vitro* cultured MSCs under a light microscope. (B) Expression of cell surface antigens, CD105, CD13 and CD45, were analyzed by flow cytometry with PE-labeled antibodies. (C) Adipogenic lineage (left panel), chondrogenic lineage (middle panel), and osteogenic lineage (right panel) differentiations of MSCs were assessed by Oil Red staining, Alcian Blue staining, and Alizarine Red staining, respectively.

Suspended THP-1 monocytes in our cultures were round in shape (Figure 2-2, A, left panel), and they became plastic adherent and amoeboid shape after PMA treatment (Figure 2-2, A, right panel). PMA-induced macrophages (Figure 2-2,

B, right panel) showed high phagocytic capability for latex beads compared with unstimulated THP-1 monocytes (Figure 2-2, B, left panel). The expression levels of the macrophage-differentiation markers CD36, CD11b and CD14, were all up-regulated in the PMA-induced macrophage group as compared to the THP-1 monocyte group (Figure 2-2, C). Based on the evidence of cell morphology, phagocytic capacity, and surface markers, we confirmed that THP-1 monocytes were induced into macrophages by PMA.

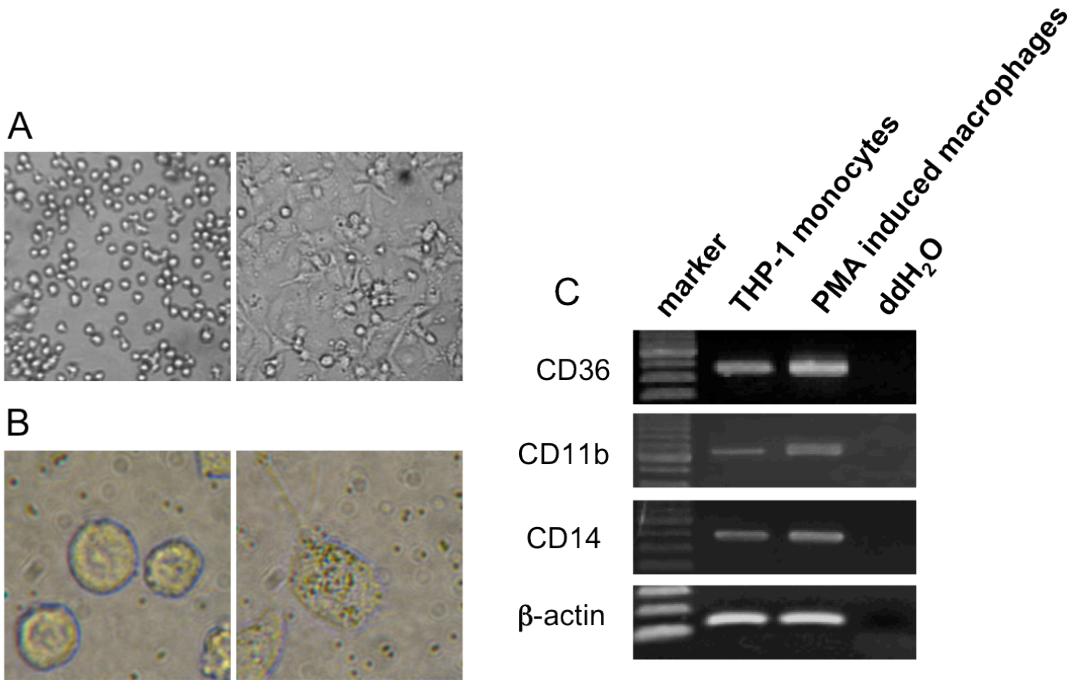

Figure 2-3. (A) Morphology of monocytes (left) and PMA-induced macrophages (right). (B) Phagocytosis of latex beads by monocytes (left) and macrophages (right). (C) Up-regulation of macrophage differentiation markers CD36, CD11b, and CD14 was observed in the PMA-induced macrophage group. Human β-actin was used as the endogenous reference gene.

2.3.2 Suppressed migration of MSCs by macrophages

The effect of macrophages on the migration capacity of MSCs is shown in Figure 2-3. There was no difference in migration capacity of MSCs between

groups treated with monocyte CM and basal medium (Figure 2-3, A and B). However, the number of migrated MSCs was significantly reduced by 36% in the group treated with macrophage CM as compared with groups treated with monocyte CM and basal medium, respectively (Figure 2-3, B, both $p<0.001$). In addition, a concentration-dependent step-wise inhibition of macrophage CM on MSCs migration was observed (Figure 2-3, C, $p<0.001$).

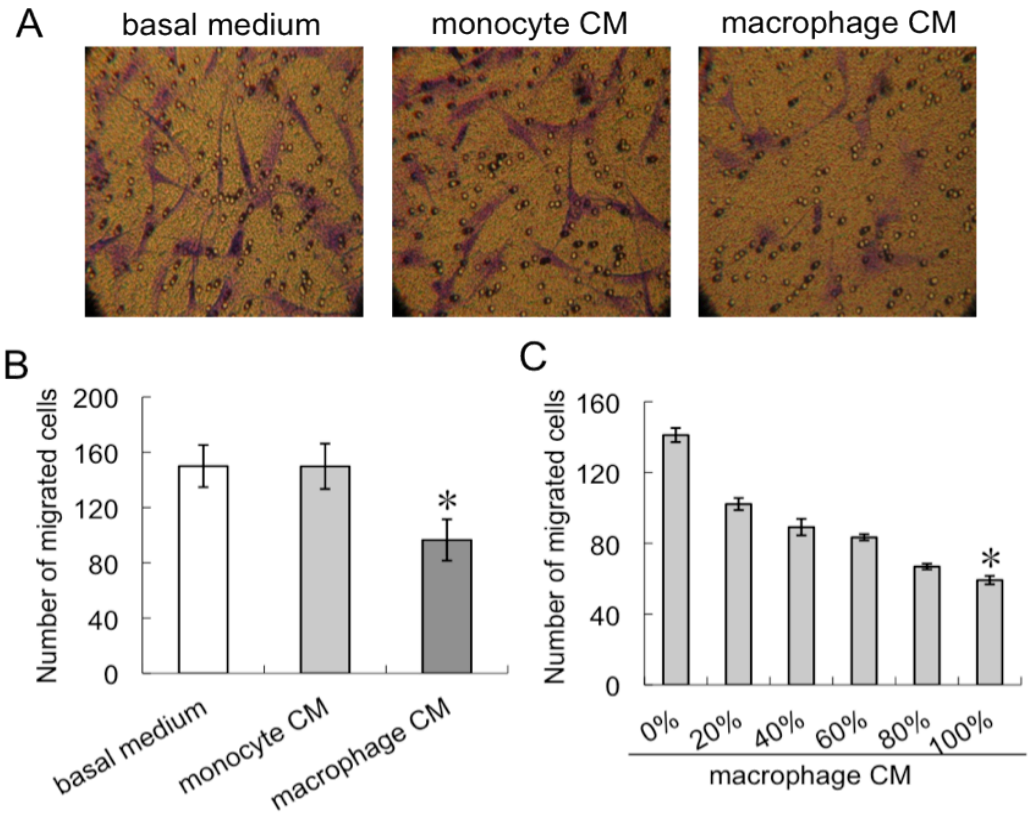

Figure 2-4. (A) Crystal violet stained membrane in the cell migration assay. Representative photos of migrated MSCs treated with basal medium or 20% CM. (B) Significant reduction in the number of migrated MSCs was shown in the group treated with 20% macrophage CM as compared with two other groups. * denotes $p<0.05$ compared with the groups treated with basal medium and monocyte CM. (C) MSCs were treated with media containing various concentrations of macrophage CM. The number of migrated MSCs significantly goes down as the concentration goes up. Experiments were performed in triplicate.

2.3.3 Reduced metabolism of MSCs without changes in proliferation

MSCs metabolic activity was assessed by measuring dehydrogenases activity at day 0, day 3 and day 7 after treatment with different CM, respectively. On day 0, there was no difference in MSCs' metabolism among these three groups (Figure 2-4, A). However, significant inhibition of MSCs' metabolism was observed on day 3 and remained on day 7 in the group treated with macrophage CM as compared with groups having monocyte CM or basal medium (Figure 2-4, A, both $p<=0.001$). In addition, a significant concentration-dependent inhibition effect of macrophage CM on MSC metabolic activity was observed when treating MSCs with a range of concentration series of macrophage CM (Figure 2-4, B, $p<0.001$).

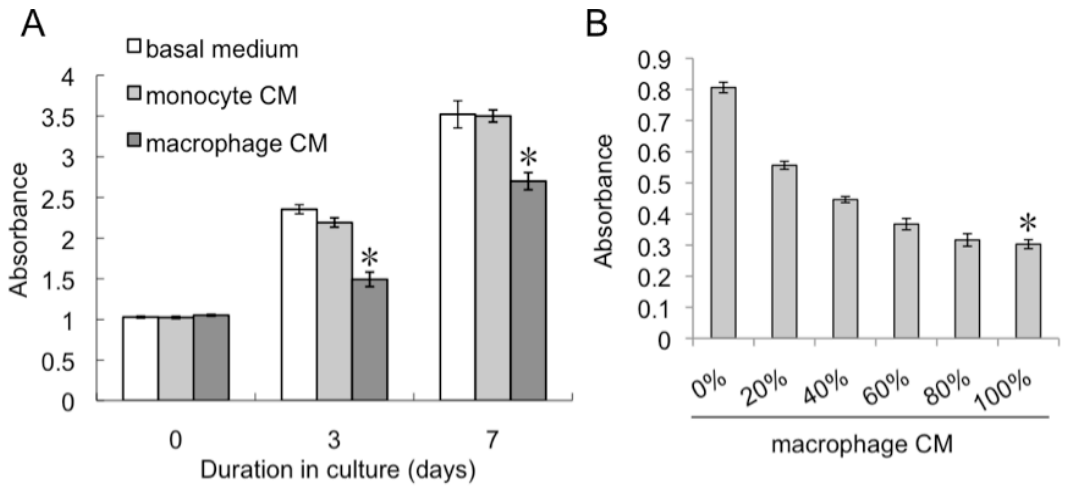

Figure 2-5. (A) 20% macrophage CM significantly decreased the metabolic activity of MSCs on day 3 and day 7 after macrophage CM treatment as compared with 20% monocyte CM and basal medium treated groups. * denotes $p<0.05$ compared with the groups treated with basal medium and monocyte CM at the same time points. (B) On day 3, macrophage CM also significantly inhibited the metabolic activity of MSCs in a concentration-dependent manner. All experiments were performed in quandraplicate.

The proliferation of MSCs was assessed by measuring DNA content after CM treatment. There was no difference among the groups treated with different media on day 0, day 3, and day 7 (Figure 2-5, A), nor was a concentration-dependent effect observed (Figure 2-5, B).

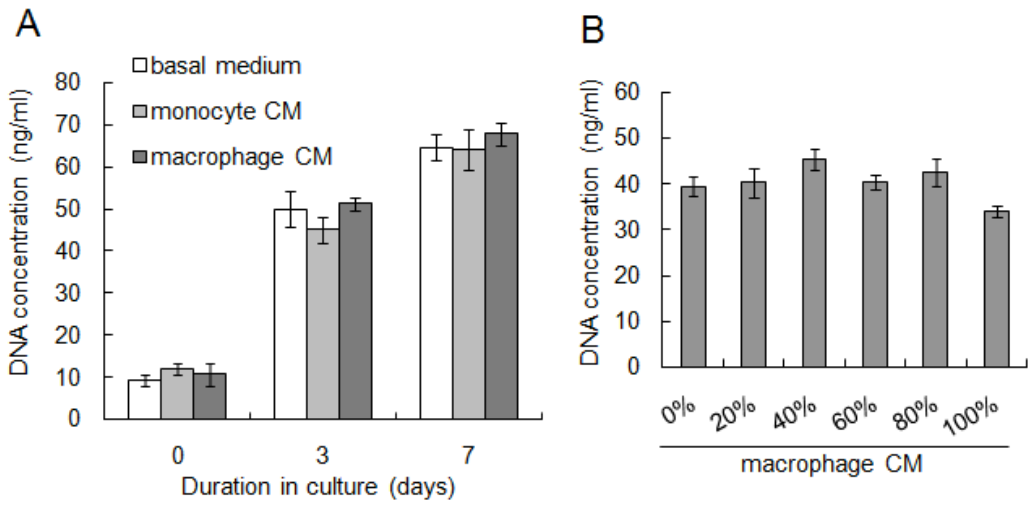

Figure 2-6. (A) Total DNA content of MSCs did not differ among groups treated with basal medium, 20% monocyte CM or 20% macrophage CM. (B) No concentration-dependent effect of macrophage CM was evident on DNA content of MSCs. Experiments were performed in quandraplicate.

2.3.4 Inhibition of osteogenic differentiation of MSCs by macrophages

The effect of macrophages on osteogenic differentiation of MSCs is shown in Figures 2-6 and 2-7. The osteogenic marker genes, ALP, IBSP, RUNX2, and OC were all significantly down-regulated in the group treated with macrophage CM for 14 days as compared with the group treated with monocyte CM (Figure 2-6, A-D, all $p<=0.02$). The significant differences between macrophage CM group and basal medium group were also observed for ALP and IBSP expression levels

(Figure 2-6, A and B, both $p<0.001$), and trend for RUNX2 and OC expression levels (Figure 2-6, C and D, $p=0.071$ and $p=0.057$, respectively).

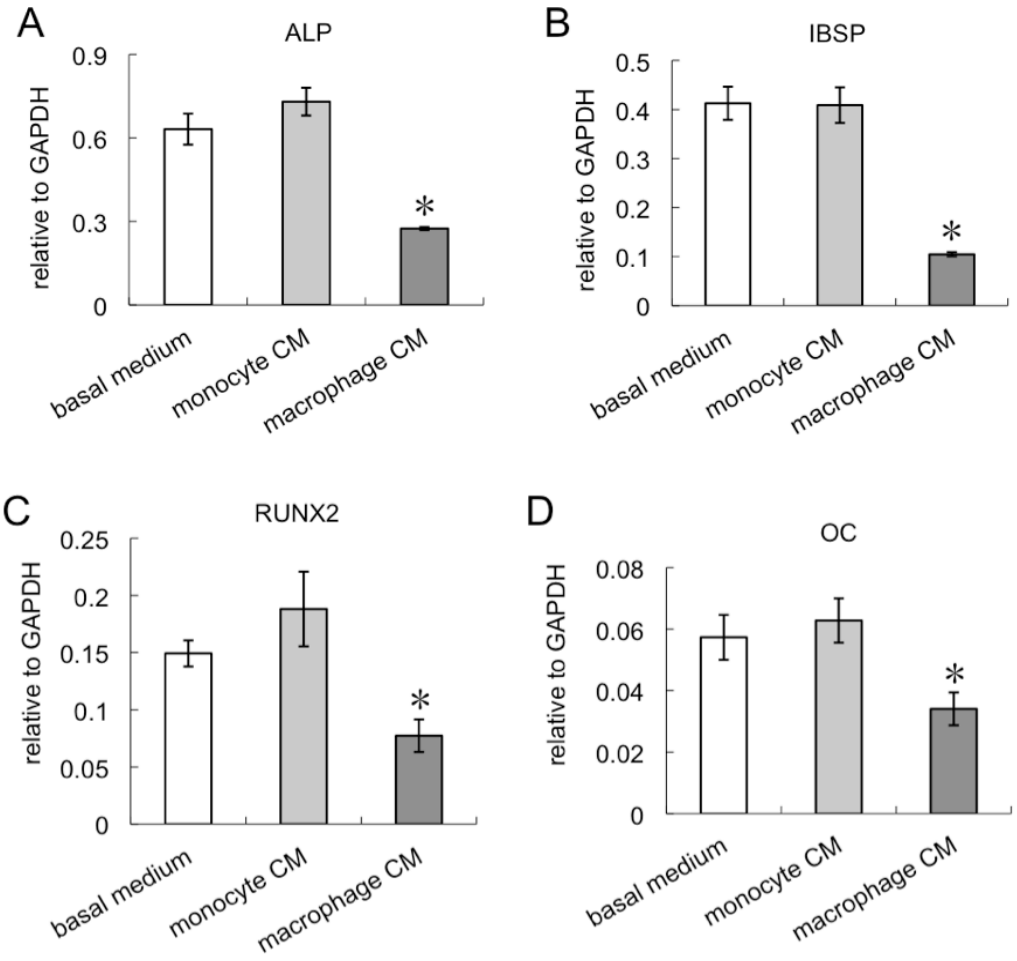

Figure 2-7. Osteogenic marker genes, ALP (A), IBSP (B), RUNX2 (C), and OC (D) were all significantly down-regulated by 20% macrophage CM treatment. Expression levels of osteogenic marker genes were assessed on the day 14 of osteogenic induction. All experiments were performed in quandraplicate.

Reduced ALP activity, assessed by Fast Blue B staining, was observed in macrophage CM treated group, as compared with two other groups (Figure 2-7, A). Macrophage CM treated MSCs showed significantly decreased ALP activity compared with those treated with monocyte CM or basal medium (Figure 2-7, B,

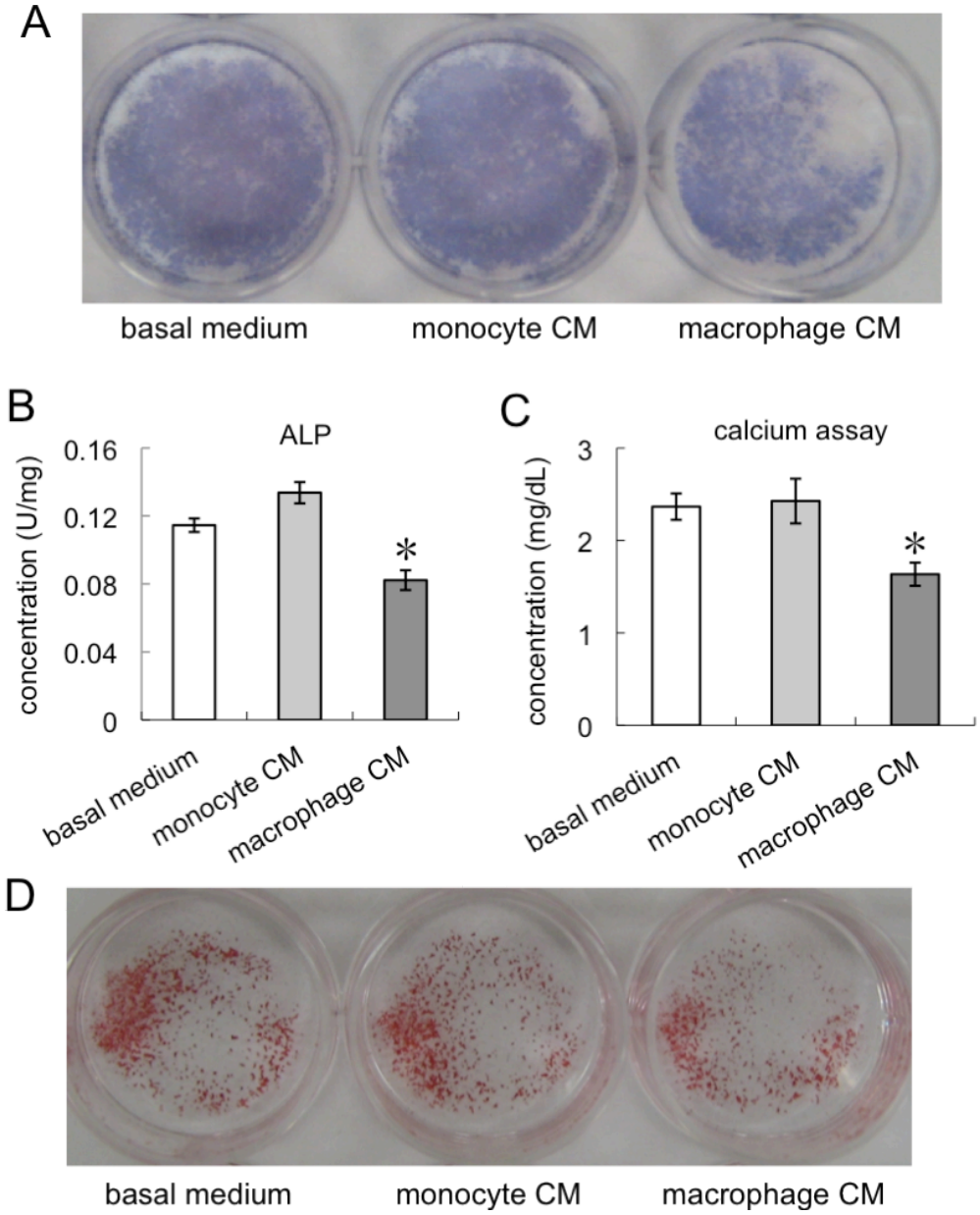

Figure 2-8. ALP activity was significantly decreased by 20% macrophage CM pretreatment, as determined by Fast Blue B staining (A), and pNPP enzymatic assay (B) on day 14. Calcium deposition was also significantly decreased by macrophage CM pretreatment, as determined by calcium assay (C) and Alizarine Red staining (D) on day 28. All experiments were performed in triplicate.

both p<=0.007). Moreover, calcium content in macrophage CM treated MSCs was significantly lower than that in monocyte CM group or basal medium group (Figure 2-7, C, both p<=0.036). Calcium staining by Alizarine Red also showed

less mineralization in macrophage CM treated group than the groups treated with monocyte CM or basal medium (Figure 2-7, D).

2.4 DISCUSSION

Although macrophages are derived from monocytes, our study shows that the conditioned medium from differentiated macrophages, but not the conditioned medium from monocytes, suppresses the migration, the metabolic activity and BMP-2-induced osteogenic differentiation of MSCs. To our knowledge, this is the first report on the effects of macrophages on osteogenesis of human MSCs.

Previous studies indicate that transplanted MSCs grafts are transiently active in host tissues, with a half-life of about 24 hours. The viability of the transplanted MSCs was poor with less than 3% of systemically administered MSCs eventually surviving in host tissues [15-18]. It is still a challenge to improve the cellular viability of MSCs after transplantation and to extend the half-life of cellular grafts. The short active life of MSCs was attributed to host immune rejection [17], but the details of the mechanism are still unknown. Our study showed that macrophage CM led to decreased dehydrogenase activity of MSCs, indicating differentiated macrophages could decrease the metabolism of MSCs. Thus, therapeutic suppression of monocyte-to-macrophage transition might be an effective way to improve the metabolic activity of transplanted MSCs graft in host tissues.

It is noteworthy that the negative regulation of macrophages on migration, metabolic activity, and BMP-2-induced osteoblast differentiation of MSCs is

unexpected, considering the positive roles of macrophages in soft tissue regeneration. For instance, in response to ischemic myocardial injury, undifferentiated splenic Ly-6C^{high} monocytes were mobilized through the blood vessels and accumulated at the injured sites where monocytes differentiated into macrophages [19]. Differentiated macrophages could phagocytise bacteria and damaged tissue debris; and in addition, they could modulate immune cells by secreting various cytokines to accelerate tissue repair [20]. This notion is supported by the observation that macrophage depletion prevents muscle membrane repair [21]. Thus, macrophages play different roles in bone fracture healing and soft tissue healing.

In normal bone fracture healing, macrophages were present in large numbers at the early stage of bone healing but became less in the bone formation area at the late stage [22]. While in delayed union and non-union fractures, immunological study indicated that CD11b positive monocytes/macrophages were consistently distributed in the connective tissue stroma with perivascular enrichment as long as 4-25 months after the bone fracture [23]. These studies indicates that macrophages are activated for a short term in normal fracture healing and remain activated for a long term in delayed union or non-union cases, suggesting that persisted activation of macrophages might be a factor associated with delayed union or non-union. This conjecture is indirectly supported by the observation that local administration of semi-soluble aminated glucan, probably through activating macrophages, led to an immature hypertrophic callus of poor

biomechanical properties in the rat femoral osteotomy model [24]. Providing the direct evidence for this concept, our study showed that differentiated macrophages suppressed migration, metabolism, and BMP-2-induced osteogenic differentiation of MSCs. So persisted macrophage activation might be one reason for delayed union or non-union.

Our study showed that macrophage CM decreased MSC migration, metabolic activity and BMP-2-induced osteogenic differentiation. It is worth of note that differentiated macrophages could secrete various products, including cytokines, growth factors, hormones, and inhibitors of enzymes, etc [25]. Among them, IL-1β, TNF-α, PDGF-AB, PDGF-BB, and TGF-β are of particular interest in that they were reported to be tightly associated with cell migration [26-29], cell proliferation [25,30,31], and osteogenic differentiation [30-32]. Some of them might play an important role in the inhibitory effects of macrophage CM on the migration, metabolic activity and osteogenic differentiation of human MSCs.

The migration capability of MSCs in our study was significantly reduced by macrophage CM as compared to monocyte CM and basal medium. Corallini and his colleague have shown that TNF-α is capable of potentiating as well as of inhibiting MSCs migration, which might result in a suboptimal recruitment of circulating MSCs in acute myocardial infarction patients [33]. Other studies reported that TNF-α alone increased the migration ability of MSCs [34-36]. Some

studies reported that PDGF-AB, PDGF-BB and BMP-4 are potent growth factors to promote migration of MSCs [27,29,37].

Both TNF-α and IL-1β are tightly associated with the bone fracture healing process. The role of TNF-α in bone fracture healing is controversial. Some studies have shown that TNF-α suppressed the expression of RUNX2 and osteocalcin of rat bone marrow MSCs [34] and inhibited bone collagen synthesis on fetal rat long bones *in vitro* [38]. However other studies have reported that TNF-α up-regulated expression of BMP-2 and ALP, and increased matrix mineralization of MSCs through NF-κB signalling pathway in human bone marrow and adipose tissue derived MSCs [39,40]. Bone marrow ablation of TNF-α receptors in mice resulted in delayed fracture healing, with lower type I collagen and osteocalcin expression as well as absent intramembranous bone formation on the periosteal surface, which indicates the TNF-α signal promotes osteogenesis during the whole fracture healing process [41]. A previous study showed that IL1-β was up-regulated at day 2 after tibia fractures and IL-1β exposure significantly decreased proliferation and osteogenic differentiation of MSCs [42], but the origin of IL1-β was unknown in that study. In mice, IL-1β inhibited proliferation and mineralization potential of MSCs [42]. IL-1β mediated the appearance and disappearance of osteoblasts, possibly by affecting the rates of differentiation and apoptosis [43].

After the bone fracture, tissue resident macrophages or macrophages differentiated from monocytes are recruited and aggregated at the fracture sites by chemotactic signals. The canonical functions of these differentiated macrophages are removing necrotic tissue debris and modulating local inflammation. Our study suggests that these macrophages inhibit the recruitment of MSCs from the bone marrow, decrease the metabolic activity of MSCs, and impair the ability of MSCs towards BMP-2-induced osteogenesis. The molecular mechanism for these observations is not clear, but cytokines and growth factors, such as TNF-α, IL-1β, PDGF-AB and PDGF-BB, which can be secreted from macrophages, might contribute to some of these effects. Further studies are warranted to identify the factors which mediate these effects.

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CHAPTER 3

NOGGIN SUPPRESSION DECREASED BMP-2-INDUCED OSTEOGENIC DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS *IN VITRO*

3.1 INTRODUCTION

New bone formation results from the osteogenic differentiation of mesenchymal stem cells (MSCs) [1]. This process is closely regulated by various signals. One of the most potent osteoinductive signals comes from bone morphogenetic proteins (BMPs) [2], which belong to transforming growth factor-β (TGF-β) superfamily of proteins. BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9 have been reported to be able to induce osteogenic differentiation both *in vitro* and *in vivo* [2,3]. BMPs elicit their signals by binding to type I receptors (BMP receptor type IA/B or activin receptor type IA) and type II receptors (BMP receptor type II or activin receptor type IIA/B) at the cell surface, and transduce signals through Smad proteins [4,5].

BMP-mediated osteogenic differentiation is tightly regulated by BMP antagonists, which include noggin, chordin, gremlin, and follistatin [6]. The function of noggin in BMP-mediated osteogenic differentiation of MSCs has been extensively investigated; however, the data are controversial. Most studies were performed in rodent cells and/or models, and the results indicated noggin to be a negative regulator of BMP-2-induced osteogenesis. In mice osteoblasts, noggin expression could be induced by BMP-2, BMP-4, BMP-6 in a time- and

dose-dependent manner, which in turn inhibited the stimulatory effects of BMPs on collagen I synthesis and alkaline phosphatase activity [7,8]. Over-expression of noggin impaired osteogenic differentiation and reduced bone formation in transgenic mice [9,10]. Masking the effect of noggin by noggin-neutralizing antibody and small interfering RNA (siRNA) increased osteogenic differentiation of murine bone marrow cells or cell line, and accelerated bone regeneration of mice with critical-sized calvarial defects [7,11,12]. These studies strongly indicate that noggin inhibits osteogenesis in rodent models. However, one study reported that noggin over-expression did not affect osteogenesis of a murine mesodermal cell line [13]. A study conducted with human MSC cultures indicated that noggin was capable of increasing alkaline phosphatase (ALP) activity and mineralization, indicating a stimulatory effect of noggin on osteogenesis of human MSCs [14]. Thus, the data regarding the effects of noggin on BMP-induced osteogenesis of MSCs are controversial. To better understand the role of noggin on human MSCs, we designed this study to evaluate the effects of knocking down noggin gene expression on BMP-2-induced osteogenesis of human bone marrow-derived primary MSCs *in vitro.*

3.2 MATERIALS AND METHODS

3.2.1 Isolation and expansion of human MSCs

Bone marrow samples were obtained from a 46-year-old male patient undergoing orthopaedic surgery after receiving the informed consent. The study was approved by the Research Ethics Committee at the University of Alberta.

Mononuclear cells from the bone marrow were isolated by centrifugation (400 g, 25 min) with Ficoll-Paque (GE Healthcare, Piscataway, NJ, USA) and then were seeded at a density of 4×10^5 cells/cm^2 in MSC growth medium (MGM: high glucose DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mg/ml Glutamax (all from Invitrogen, Carlsbad, CA, USA) and 4 ng/ml FGF-2 (Millipore, Temecula, MA, USA)). After being incubated for 3 days, non-adherent cells were discarded. Adherent cells were washed twice with phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) and expanded in MGM. After 7 days, the cells were either further expanded for experiments or frozen in 1 ml aliquots in liquid nitrogen. For all experiments, cells were incubated at 37 ^{o}C in a 5% CO_2 incubator. All the following experiments were performed in triplicates, unless otherwise indicated.

3.2.2 Effect of BMP-2 treatment on noggin expression

To determine whether BMP-2 induces noggin expression in human MSCs, we designed a dose-response study and a time-course study. For the dose-response study, MSCs were seeded in MGM in 35-mm^2 disks with 6×10^4 cells/dish. The medium was replaced with basal medium containing various concentrations (0-10 μg/ml) of BMP-2 after 24 hours. The cells were lysated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and collected 72 hours after the BMP-2 treatment. For the time-course study, MSCs in 35-mm^2 dishes were treated with or without 0.1 μg/ml BMP-2, cells samples were lysated with Trizol reagent and collected at 0, 24, 48, 72, and 96 hours after treatment.

Total RNA was extracted with phenol/chloroform method. RNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and reverse transcribed to cDNA with the iScript cDNA synthesis Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's protocol. Reverse transcription was performed in 20 μl of final volume with the following protocol: 25°C for 5 min followed by 42°C for 5 min, and 85°C for 5 min. Real-time quantitative PCR amplifications were conducted in quadruplicate with the iQ5 system (Bio-Rad, Mississauga, ON, Canada). The 25 μl reaction mixture contained 10 ng cDNA from reverse transcription of an individual sample, 200 nM of each primer, and 1× iQ SYBR Green supermix (Bio-Rad, Mississauga, ON, Canada). Real-time quantitative PCR was performed with the following protocols: one cycle of 95°C for 3 min, followed by 45 cycles of 95°C for 10 sec, 58°C for 20 sec, and 72°C for 10 sec. Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as the endogenous reference gene to normalize noggin gene expression. All primers (Invitrogen, Carlsbad, CA, USA) used in this study were listed in Table 3-1.

Table 3-1. Sequences of PCR primers used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
GAPDH	GGACTCATGACCACAGTCCAT	CAGGGATGATGTTCTGGAGAG
Noggin	GGAGGAAGTTACAGATGTGGCTGT	CACTCGGAAATGATGGGGTACTG
ALP	ACTCCCACTTCATCTGGAACC	CCTGTTCAGCTCGTACTGCAT
IBSP	ACAGGGTTAGCTGCAATCCA	TGCCTTGTTCGTTTTCATCC
OC	GAAGCCCAGCGGTGCA	CACTACCTCGCTGCCCTCC
RUNX2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA

3.2.3 Optimization of siRNA transfection condition

To identify the best condition for siRNA transfection, we selected several combinations of siRNA duplex with the transfection reagent (Table 3-2) and tested the transfection efficiency by flow cytometry. BLOCK-iT Alexa Fluor Red fluorescent siRNA (referred as fluorescent siRNA in the following text, Invitrogen, Carlsbad, CA, USA) is an Alexa Fluor 555-labeled RNA duplex with the same length, charge, and configuration as standard siRNA. In this study it was transfected into MSCs with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) to optimize the transfection condition. MSCs were seeded in 12-well plates (BD Biosciences, Franklin Lakes, NJ, USA) with 2×10^4 cells per well. After 24 hours, medium was replaced with 1 ml MGM without antibiotics. Fluorescent siRNA and RNAiMAX were diluted separately in Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA). Diluted fluorescent siRNA and diluted RNAiMAX were combined, gently mixed, and incubated for 20 min at room temperature. Then, 200 μl fluorescent siRNA-RNAiMAX complex was added to each well. 24 hours later, the cells were washed with cold PBS and detached with 0.05% trypsin. The percentage and mean fluorescence intensity of transfected MSCs was analyzed by using a Quanta SC Flow Cytometer (Beckman Coulter, Mississauga, ON, Canada) with the excitation light of 488 nm and emission peak of 575 nm. The transfection system that yielded the highest percentage and highest mean fluorescence intensity of transfected MSCs was chosen as the optimal one.

Table 3-2. Combination of reagents used for transfection

Groups	Fluorescent siRNA (nM, final concentration)	RNAiMAX (μl)
No transfection	0	0
Fluorescent siRNA	5	0
	16.6	0
RNAiMAX	0	1
	0	3
Fluorescent siRNA/RNAiMAX	5	1
	5	3
	16.6	1
	16.6	3

3.2.4 Analysis of noggin siRNAs efficacy

Noggin expression was knocked down with human noggin siRNAs (Qiagen, Mississauga, ON, Canada), which were designed and synthesized based on the sequence of human noggin (Gene Accession Number: NM_005450). Sequence information for noggin siRNAs was listed in Table 3-3. Preparation of siRNA-RNAiMAX complex and transfection were conducted according to the optimized transfection system.

Table 3-3. Information about noggin siRNAs

siRNA name	Qiagen Catalogue number	Sequence (5'-3')	
noggin siRNA1	SI00077392	Target	AAGGTCAGTATTATACGTTAA
		Sense	GGUCAGUAUUAUACGUUAA
		Antisense	UUAACGUAUAAUACUGACC
noggin siRNA2	SI03086643	Target	CGGCTGGATTCCCATCCAGTA
		Sense	GCUGGAUUCCCAUCCAGUATT
		Antisense	UACUGGAUGGGAAUCCAGCCG
noggin siRNA3	SI00077399	Target	CCGAGCGAGATCAAAGGGCTA
		Sense	GAGCGAGAUCAAAGGGCUATT
		Antisense	UAGCCCUUUGAUCUCGCUCGG

We tested three noggin siRNAs which target to different regions of noggin cDNA. The fluorescent siRNA has the same length, charge, and configuration as standard siRNA and its sequence is not homologous to any known gene.

Therefore, we used it as the control siRNA. To validate the knockdown efficacy of these three noggin siRNAs, MSCs were transduced with fluorescent siRNA (control siRNA) or noggin siRNAs under the previously optimized transfection condition. After a 24-hour transfection, MSCs were exposed to basal medium containing 0.1 μg/ml BMP-2. The total RNA was extracted 72 hours after treatment and noggin expression was assessed by quantitative real-time PCR as described above.

3.2.5 Analysis of noggin siRNA duration

To further understand the duration of noggin down-regulation by siRNA in the presence of BMP-2, we conducted a time-course study on noggin expression after siRNA transfection. Before transfection, we collected samples as the baseline of noggin expression (named as day 0). For the transfection, we set up four groups in triplicate: 1) MSCs with no transfection of siRNA (named as NT group), 2) MSCs transfected with non-targeting negative control siRNA (named as control siRNA group), 3) MSCs transfected with noggin siRNA1 (named as NOGsi1 group), and 4) MSCs transfected with noggin siRNA3 (named as NOGsi3 group). After a 24-hour transfection, medium was replaced with basal medium containing 0.1 μg/ml BMP-2, and from then on, cells were collected on the days 3, 7, 10, 14, and 21. Total RNA extraction and quantitative real-time PCR were performed to assess the expression levels of noggin at the indicated time points.

3.2.6 Induction of osteogenic differentiation of human MSCs

To understand the effect of noggin suppression on osteogenic differentiation of MSCs, we knocked down noggin expression in MSCs and then induced MSCs to undergo osteogenic differentiation with basal medium containing 0.1 μg/ml BMP-2 in 12-well plates. There were four study groups: NT group, control siRNA group, NOGsi1 group, and NOGsi3 group. Since an initial study indicated efficacy of noggin siRNA to last for at least 7 days (Figure 3-3 B), we performed siRNA transfection every 7 days.

3.2.7 Real-time quantitative PCR analysis of osteogenic marker genes

On the day 14 of osteogenic induction, cell samples were collected, and total RNA were extracted and reverse transcribed as described above. Expression levels of osteogenic marker genes, alkaline phosphatase (ALP), integrin-binding sialoprotein (IBSP), runt-related transcription factor-2 (RUNX2), and osteocalcin (OC), were examined by real-time quantitative PCR. Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was used as the endogenous reference gene to normalize the target gene expression. Real-time quantitative PCR were performed as described above. All primers were listed in Table 3-1.

3.2.8 Alkaline phosphatase assay

On the day 14 of osteogenic induction, ALP activity was quantitatively measured by a colorimetric assay (BioAssay Systems, Hayward, CA, USA). The cells in triplicate cultures were lysated with buffer containing 0.5% Triton, 50

mM Tris-HCl, and 5 mM $MgCl_2$. The lysate was then transferred to 96-well plates, incubated with p-nitrophenylphospate substrate at 37°C for 30 min and then the reaction was halted with the stop buffer. The p-nitrophenol product was then measured at 405 nm using the microplate reader (BioTek, Winooski, VT, USA). Protein concentration of the cell lysate was measured with a DC Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada) according to the manufacturer's instructions, and bovine serum albumin (BSA, Bio-Rad, Mississauga, ON, Canada) was used to create a standard curve to transform data. ALP concentration was normalized by the total protein amount.

3.2.9 Alkaline phosphatase staining

On the day 14 of osteogenic induction, ALP staining was performed with a Fast Blue B Kit (Sigma, St. Louis, MO, USA). Briefly, samples were washed three times with PBS and fixed with citrate-acetone-formaldehyde fixative for 30 sec. After a brief rinse with deionized water, samples were stained with sodium nitrite/FBB alkaline solution in the dark for 15 min, and counterstained with neutral red solution for 2 min. Samples were then washed three times with tap water to remove the dissociative dye.

3.2.10 Calcium deposit assay

On the day 28 of osteogenic induction, the cells and extra-cellular matrix were lysated and demineralized by adding 1 ml of 0.5N HCl to each well of 12-well plates and incubating at 4°C overnight. The supernatant containing calcium

extracts was collected after centrifugation at 10,000 g for 10 min. The calcium concentration was measured with a QuantiChrom Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA) according to manufacturer's instructions. Higher absorbance value indicates higher calcium concentration.

3.2.11 Alizarin red staining

On the day 28 of osteogenic induction, cells and the extracellular matrix were fixed with 70% alcohol, rinsed rapidly in distilled water, and stained by Alizarin red S solution (Sigma, St. Louis, MO, USA) for 30 sec to 5 min until orange-red color. After moving off excess dyes, 20 dips acetone and 20 dips acetone-xylene (Sigma, St. Louis, MO, USA) were added separately, followed by clearing in xylene.

3.2.12 Water soluble tetrazolium salt-8 (WST-8) assay

MSCs were seeded in 48-well plates at a density of 5×10^3 cells per well in MGM without antibiotics. 24 hours later, MSCs in 300 μl medium were transfected with control siRNA, noggin siRNA1, or noggin siRNA3 (60 μl transfection mixture). WST-8 assay was performed before transfection (0d) and 3 days after transfection (3d). A Cell Counting Kit-8 (Cedarlane, Burlington, ON, Canada) was used to count living cells by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay. Briefly, 30 μl of the WST-8 solution was added to the 300 μl medium, and incubated for additional 3 hours. Absorbance was measured with the microplate

reader at 450 nm with a reference wavelength of 650 nm. Higher absorbance indicates more living cells.

3.2.13 DNA assay

MSCs were seeded in 96-well plates at a density of 1×10^3 cells per well and cultured in the 100 μl MGM without antibiotics. 24 hours later, MSCs were transfected with control siRNA, noggin siRNA1 or noggin siRNA3 (20 μl transfection mixture) for 24 hours. On the day before transfection (0d) and 3 days after finishing transfection (3d), cells were lysated and samples were collected. The DNA content was measured with a CyQUANT Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Fluorescence was read with the microplate reader with the excitation light of 450 nm and emission wavelength of 530 nm. Higher fluorescence stands for higher DNA content. Bacteriophage λDNA of known concentrations was used to create the standard curve to transform data.

3.2.14 Statistical analysis

Data was presented as the mean with the standard deviation and analyzed by one-way ANOVA followed by Bonferroni post-hoc test. All tests were 2-sided with $p<0.05$ considered as the level of significance. All analyses were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

3.3 RESULTS

3.3.1 BMP-2 induced noggin expression in a dose- and time-dependent manner in human MSCs

A clear dose-dependent induction of noggin by BMP-2 was observed (Figure 3-1, A, $p<0.001$). Noggin mRNA expression of MSCs was significantly up-regulated by 0.1, 1, 5, and 10 μg/ml BMP-2 (Figure 3-1, A, all $p<=0.002$ compared with the group without BMP-2 treatment), but not by 0.01 μg/ml BMP-2 (Figure 3-1, A, $p=0.321$ compared with the group without BMP-2 treatment). There was no significant difference of noggin expression between the group treated with 0.1 μg/ml BMP-2 and the groups treated with 1, 5, 10 μg/ml BMP-2 (Figure 3-1, A, all $p>0.462$); therefore, 0.1 μg/ml is the lowest identified dosage of BMP-2 which could up-regulate noggin expression in human MSCs.

For the time-course study, without BMP-2 treatment, noggin expression slightly increased at 48 h and 72 h, and became significantly higher at 96 h compared with that of 0 h (Figure 3-1, B, $p<0.001$). Noggin expression was significantly up-regulated by 0.1 μg/ml BMP-2 treatment at 72 h and 96 h (Figure 3-1, B, $p=0.011$ and $p<0.001$, respectively), compared to the group without BMP-2 treatment at the same time point.

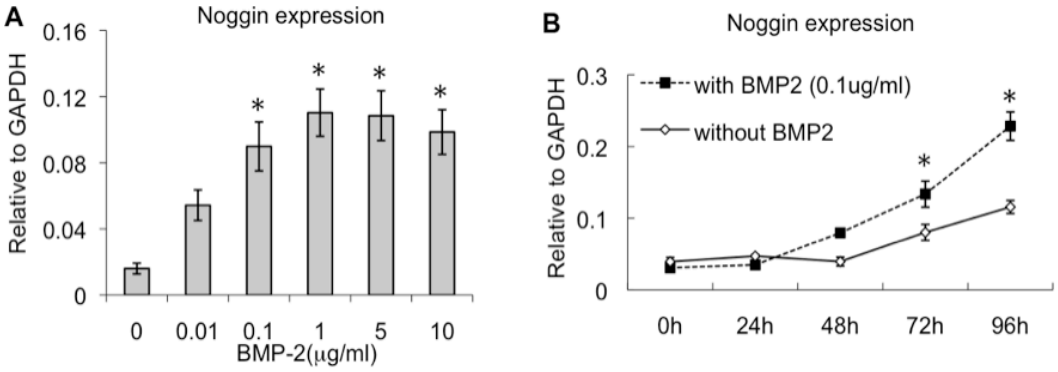

Figure 3-1. Dose- and time-dependent effects of BMP-2 on noggin expression. (A) expression of noggin transcript after treating with various dosages of BMP-2 for 3 days. * denotes $p<0.05$ compared with the noggin expression level of no BMP-2 treatment group. (B) expression of noggin transcript at various time points after treating with 0.1 μg/ml BMP-2. * denotes $p<0.05$ compared with noggin expression level of the group without BMP-2 treatment at the same time point.

3.3.2. Optimal transfection condition

Among the transfection conditions we examined, the best one for MSCs transfection turned out to be 16.6 nM of siRNA duplex (final concentration) with 3 μl Lipofectamine RNAiMAX in 1200 μl medium for 12-well plates. After a 24-hour transfection, this transfection condition yielded the highest percentage of fluorescence positive cells, about 80% (Figure 3-2, A). In addition, this transfection condition also achieved the highest mean fluorescence intensity of transfected cells, among all the transfection conditions (Figure 3-2, B).

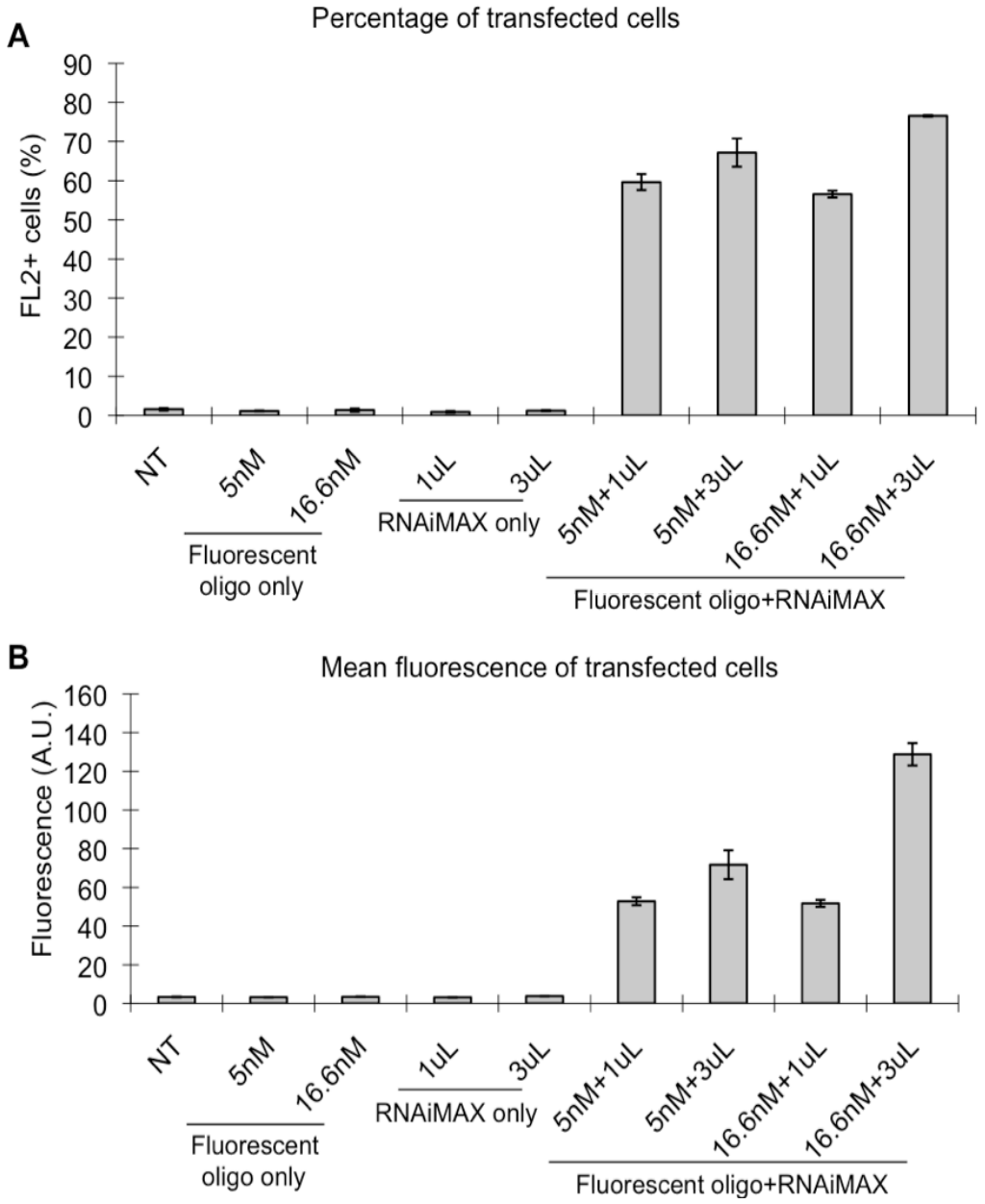

Figure 3-2. (A) Percentage of fluorescence-labeled MSCs in various transfection conditions was assessed after a 24-hour transfection. (B) Mean fluorescence of transfected MSCs was analyzed in the same transfection conditions. A.U. stands for arbitrary unit. Experiments were performed in triplicate.

3.3.3 The efficacy of noggin siRNAs and the duration of suppression effect

Transfection of control siRNA did not affect noggin expression (Figure 3-3 A). Noggin siRNA1 and noggin siRNA3 significantly reduced the expression level of noggin transcripts (Figure 3-3 A, both $p<0.001$ vs. NT group and control siRNA group), while noggin siRNA2 was not able to decrease noggin expression (Figure 3-3 A).

Noggin siRNA1 and noggin siRNA3 were able to knock down noggin from the day 3 to day 7 after transfection (Figure 3-3 B, all $p<0.014$ vs. NT group and control siRNA group). 10 days after transfection, there was no significant difference among groups. Noggin siRNA3 slightly decreased noggin expression on the day 14 after transfection, though the difference was not significant (Figure 3-3 B, $p>=0.155$ vs. both NT group and control siRNA group). Unexpectedly, expression levels of noggin were significantly increased by noggin siRNA1 and noggin siRNA3 on the day 21 after transfection, compared with NT group and control siRNA group (Figure 3-3 B, both $p<=0.05$).

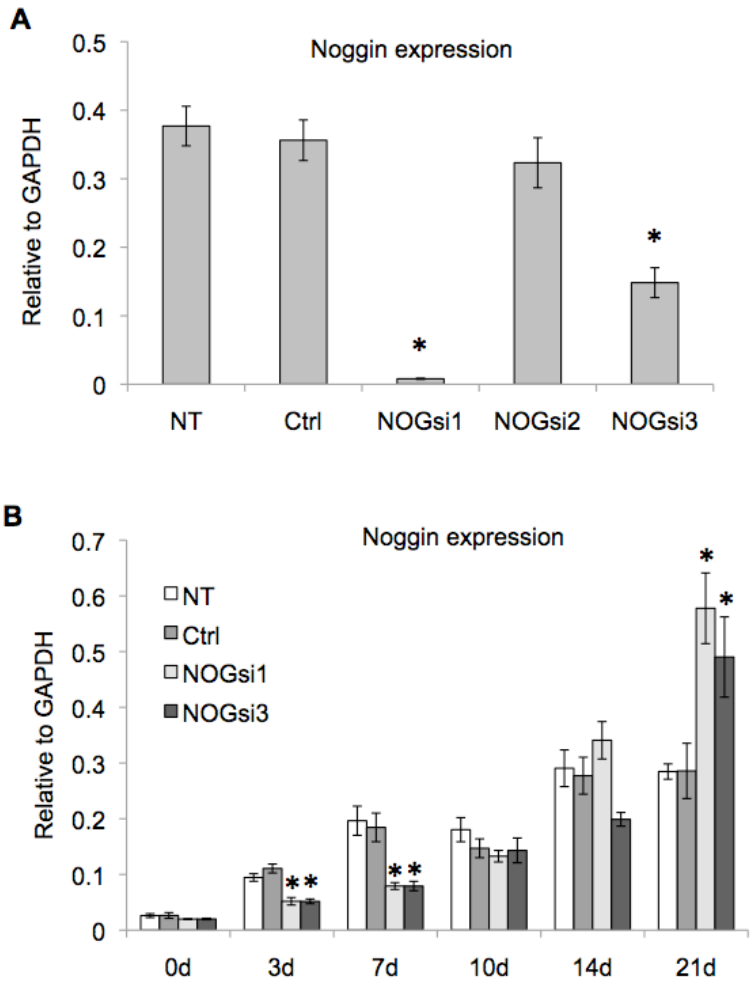

Figure 3-3. Efficacy and effect duration of noggin siRNAs. (A) Assessment of efficacy of noggin siRNAs 3 days after siRNA transfection. * denotes $p<0.05$ compared with NT group (NT) and control siRNA group (Ctrl). Noggin siRNA# was shortened as NOGsi#. (B) Assessment of suppression duration of noggin siRNAs. MSCs were cultured in basal medium containing 0.1 μg/ml BMP-2 after a single transfection. Noggin expression level was assessed at various time points. * denotes $p<0.05$ compared with NT group (NT) and control siRNA group (Ctrl) at the same time point. Experiments were performed in triplicate.

3.3.4 Decreased osteogenesis of MSCs after constant noggin suppression

ALP expression was slightly down-regulated by noggin siRNA1 and noggin siRNA3, though the difference was not significant (Figure 3-4, A, both $p>=0.34$

vs. NT group and control siRNA group). IBSP was significantly down-regulated by noggin siRNA1 and noggin siRNA3, compared with the NT group and control siRNA group (Figure 3-4, B, both $p<0.001$). Expression of OC was also significantly down-regulated by noggin siRNA1 and noggin siRNA3 (Figure 3-4, C, both $p<=0.038$ vs. NT group and control siRNA group). Expression of RUNX2 gene was not significantly affected by noggin siRNA1 and noggin siRNA3 (Figure 3-4, D, both $p>=0.305$ vs. NT group and control siRNA group).

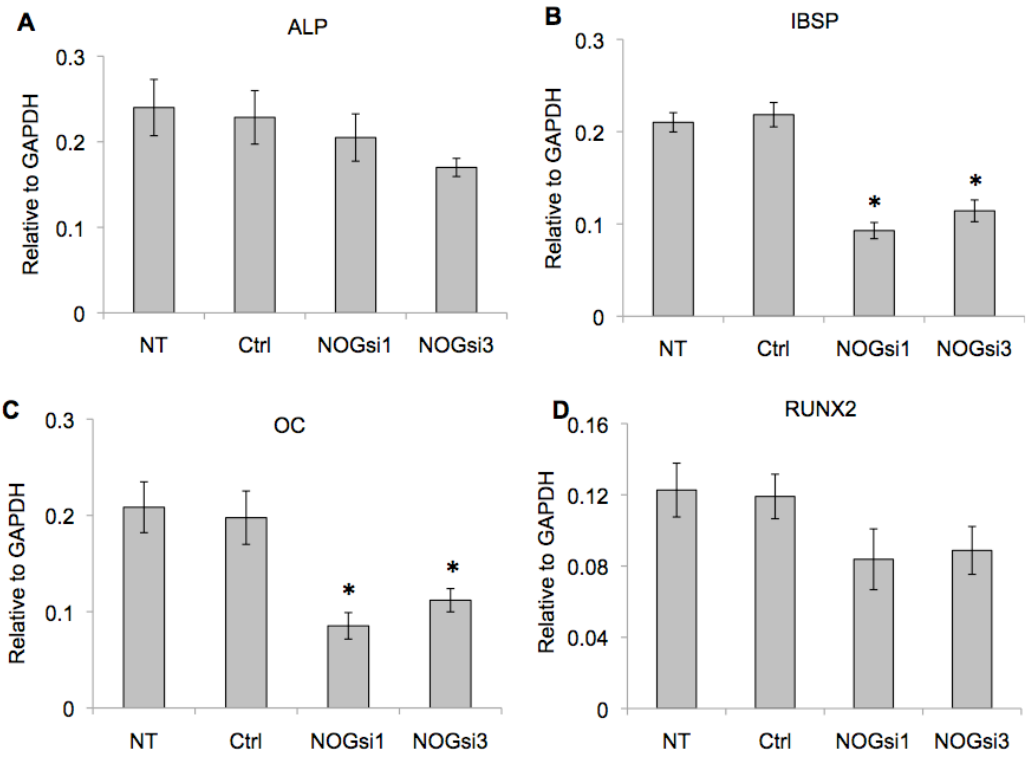

Figure 3-4. Expression of osteogenic marker genes, ALP (A), IBSP (B), OC (C), and RUNX2 (D) was assessed 14 days after osteogenic induction by 0.1 μg/ml BMP-2. * denotes $p<0.05$ compared with NT group (NT) and control siRNA group (Ctrl). Experiments were performed in quandraplicate.

ALP assay indicated that noggin siRNA1 and noggin siRNA3 could significantly decrease ALP activity, compared with NT group (Figure 3-5, A, both p<=0.024). The same pattern was observed in ALP staining by Fast Blue B staining (Figure 3-5, B).

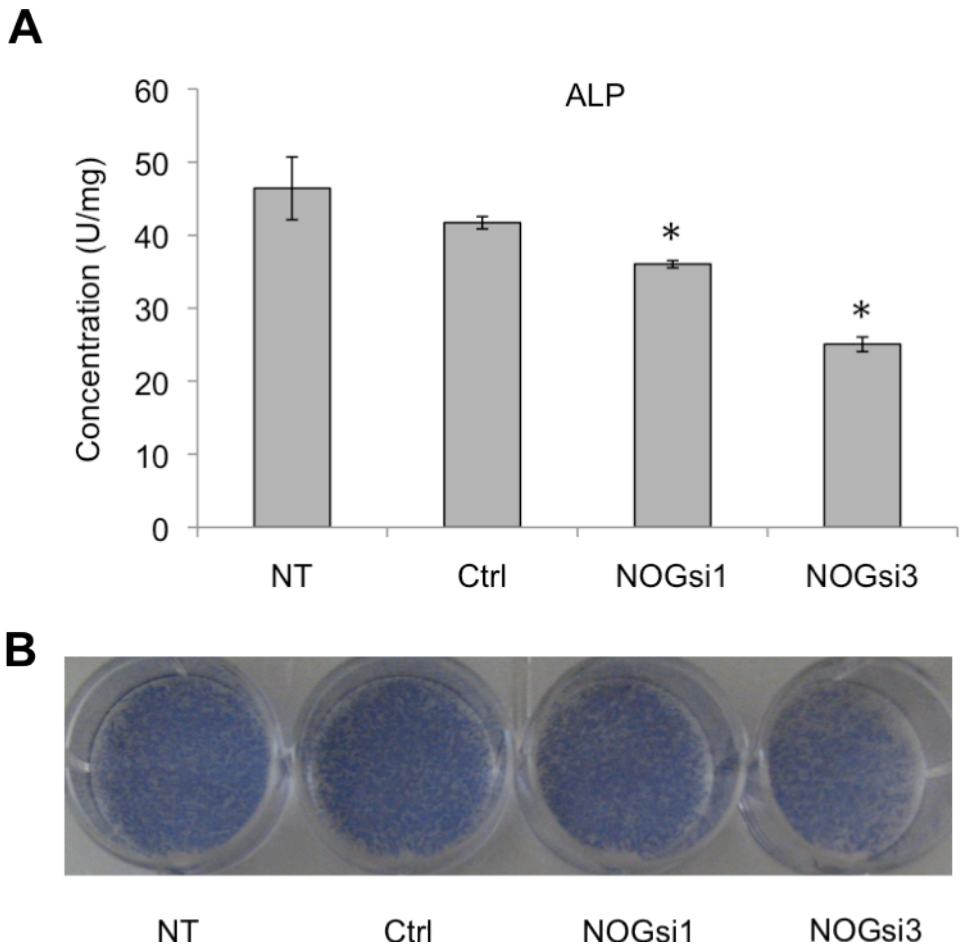

Figure 3-5. Noggin suppression decreased ALP activity. ALP activity was decreased by noggin siRNAs as determined by pNPP enzymetic assay (A) and Fast Blue-B staining (B) on the day 14 of osteogenic induction by 0.1 μg/ml BMP-2. * denotes p<0.05 compared to NT group. Experiments were performed in triplicate.

Calcium deposition assay also indicated that noggin siRNA1 and noggin siRNA3 significantly decreased calcium deposition, as compared with the NT

group (Figure 3-6, A, both p<=0.048). Alizarine Red staining also showed less calcium deposits were produced in the groups transfected with noggin siRNA1 and noggin siRNA3, compared with two other groups (Figure 3-6, B).

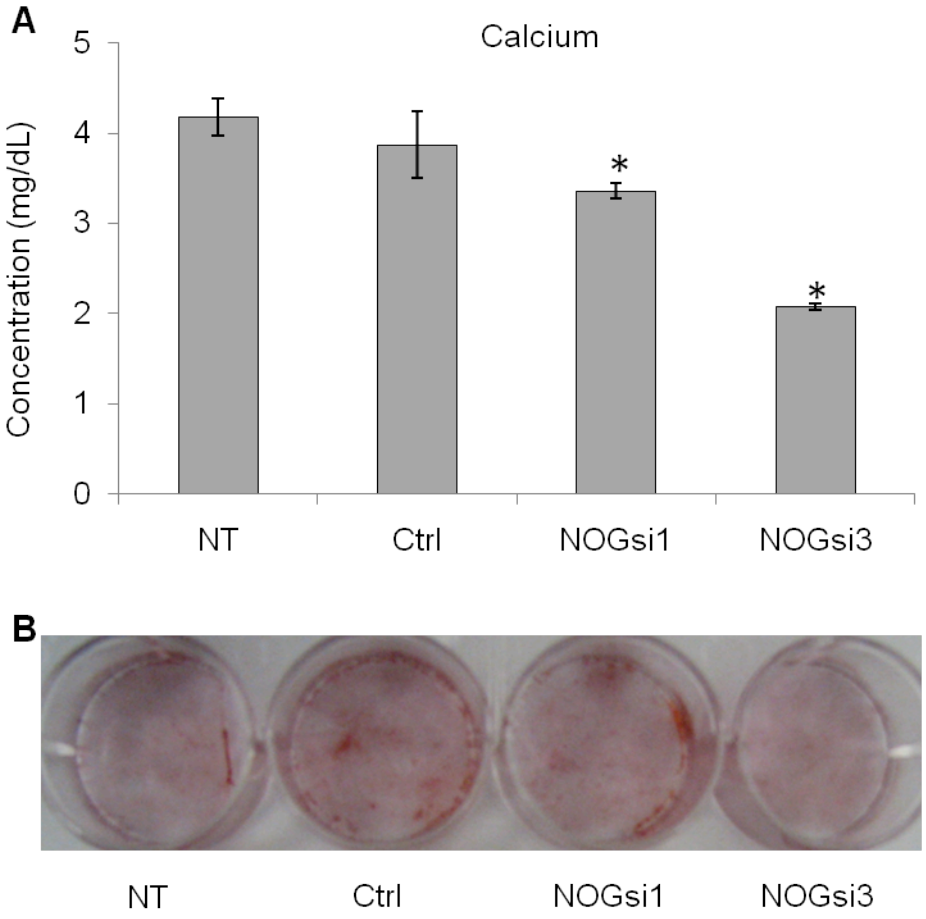

Figure 3-6. Calcium deposit was decreased by noggin siRNAs, as determined by calcium assay (A) and Alizarine Red staining (B) on the day 28 of osteogenic induction by 0.1 μg/ml BMP-2. * denotes p<0.05 compared to NT group. Experiments were performed in triplicate.

3.3.5 Decreased proliferation of MSCs after noggin suppression

WST-8 assay indicated that MSC metabolic activity was significantly reduced by noggin siRNA1 and siRNA3 3 days after transfection (Figure 3-7 A, both p<0.001 vs. NT group and control siRNA group). DNA assay showed that the

total DNA content was significantly decreased by noggin siRNA1 on the day 3 after transfection, compared with NT group and control siRNA group (Figure 3-7 B, both p<=0.001).

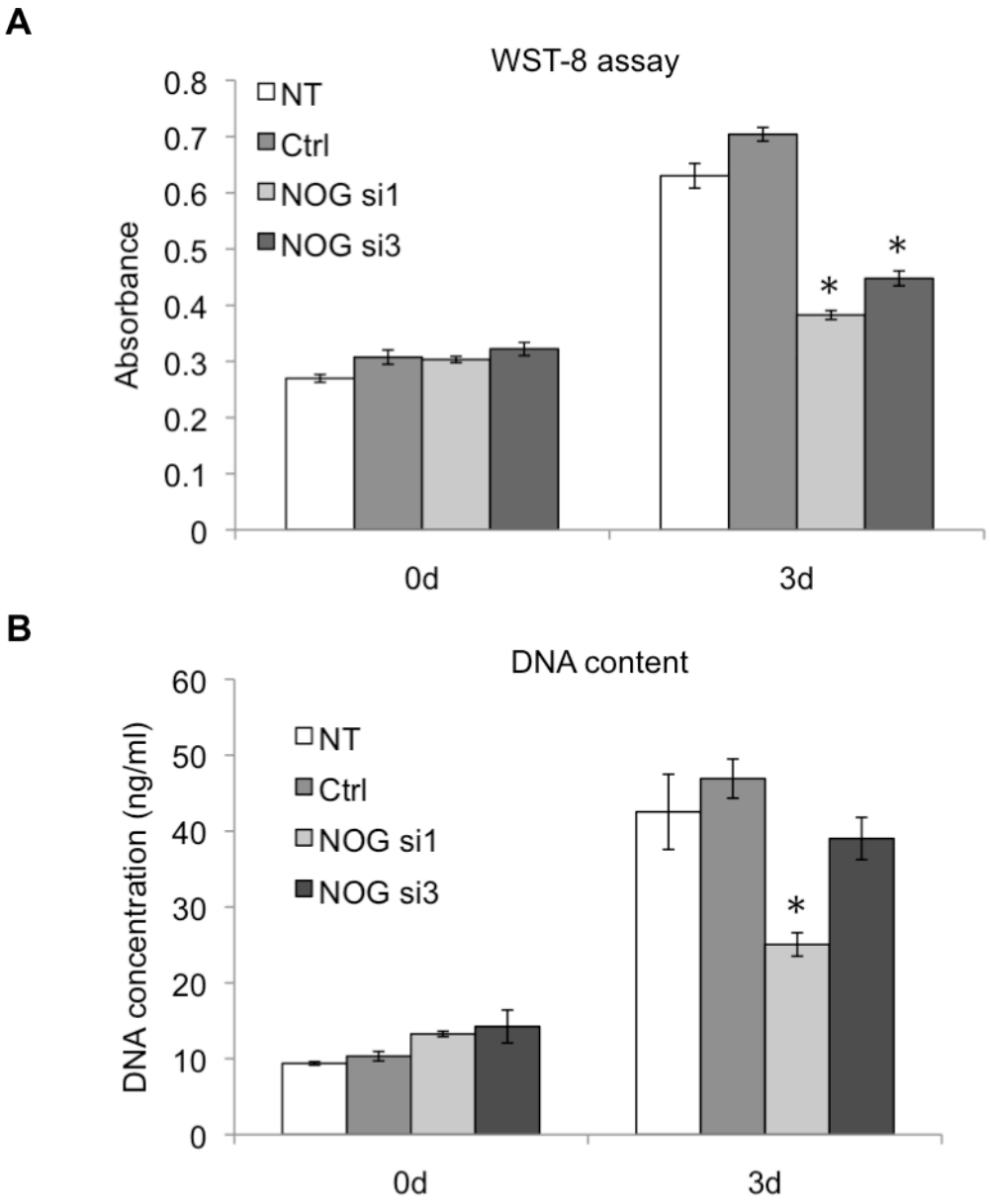

Figure 3-7. Noggin suppression decreased the metabolism and DNA content of human MSCs. (A) WST-8 assay for MSCs under different treatments. (B) DNA content of MSCs under different treatments. * denotes p<0.05 compared with NT group (NT) and control siRNA group (Ctrl) at the same time point. Experiments were performed in quandraplicate.

3.4 DISCUSSION

In the present study, effects of noggin suppression on osteogenesis of human MSCs were assessed. We found noggin expression was up-regulated by BMP-2 treatment in a dose- and time-dependent manner in human MSCs. Noggin siRNA could knock down expression of noggin transcript for at least 7 days. We found noggin suppression inhibited BMP-2-induced osteogenesis of MSCs, as demonstrated by down-regulated expression of osteogenic marker genes, decreased ALP activity and less calcium deposits. In addition, noggin suppression decreased the metabolic activity and DNA content of MSCs. These results indicate that expression of noggin might have stimulatory effects on osteogenesis of human MSCs as well as viability. This study, by RNA interference, added more knowledge about the roles of noggin in BMP-2-induced osteogenesis of human MSCs.

These observations in human MSCs are diametrically opposite to many studies in the murine cell lines and animal models [11,12,15]. Endogenous expression of noggin was observed in the rodent model of fracture healing [16]. Skeletal-specific overexpression of noggin in mice decreased expression of osteogenic marker genes, trabecular bone volume, and bone formation rates [9,10]. Besides, noggin suppression by siRNA enhanced *in vitro* osteogenesis and accelerated *in vivo* bone formation in mice [11,12]. These data indicate that noggin is the negative regulator of bone fracture healing in mice. However, our observation is in agreement with one previous study conducted with human MSC cultures. In that

study, the addition of noggin in cell culture could induce ALP, increase mineralization, and up-regulate expression of several osteogenic marker genes [14], which indicates that noggin is a positive regulator in osteogenesis of human MSCs. However, our study does not support that noggin did not affect osteogenic differentiation as observed in a murine teratocarcinoma-derived mesodermal cell line [13].

These contradictory results reflect that there might be a species-specific difference in the role of noggin during BMP-2-induced osteogenesis of MSCs. This conjecture is supported by previous observations that BMP-2 and DEX exerted different osteoinductive effects on MSCs from humans, rats, and mice [17,18]. It's also reported that though rat MSC expressed mRNA for ALK-6 (type I BMP receptor), human MSCs lack this particular receptor [18]. BMP-2 could up-regulate Msx-2, a transcription factor which inhibit osteogenesis of osteoprogenitor cells, up to 10 folds in human MSCs, but BMP-2 did not change Msx-2 expression in rat MSCs [18]. These observations indicate that there is a species-specific difference during BMP-2-induced osteogenesis of MSCs.

In the present study, we also observed that noggin suppression decreased the metabolic activity and DNA content of human MSCs. This may indicate that noggin might be also crucial for proliferation of human MSCs. It was reported that noggin maintains pluripotency of human embryonic stem cells (ESCs) and increased proliferation of human ESCs [19,20]. Noggin and basic FGF synergically

suppressed BMP signaling and sustained the undifferentiated proliferation of human ESCs [21]. In addition, in murine hippocampal subgranular zone, noggin retained the self-renewal and increased the proliferation of neural stem cells [22]. Collectively, noggin seems to play important roles in the maintenance and proliferation of stem cells.

We conclude that the noggin expression in human MSCs culture was up-regulated by BMP-2 in a dose- and time-dependent manner. Noggin suppression decreased the BMP-2-induced osteogenic differentiation and proliferation of human MSCs. Our results, contrary to the extensive studies conducted in rodent cells and animal models, corroborated with one previous study on human MSCs that the addition of noggin in the cell culture increased osteogenesis of human MSCs. This suggests that the effects of noggin on BMP-2-induced osteogenesis of MSCs might be species-specific.

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

4.1 GENERAL DISCUSSION

Bone fracture is one of the most common cases in the current orthopedic practice. It has been reported that more than 6.2 million cases of bone fracture occur in United States annually and about one in ten patients will develop into delayed union or non-union, which significantly decrease patients' postoperative quality of life. To facilitate the bone fracture healing, various therapy intervention methods have been developed. Currently, bone grafting and *in situ* administration of bone morphogenetic proteins (BMPs) are two most common methods used in clinical setting to treat none-union and aid spinal fusion. Bone grafts with exception of autologous bone graft show remarkable limitations in osteoconductive, osteoinductive or osteogenic activity. While autologous bone grafting is the gold standard with best clinical effectiveness, it has limited volume and is associated with morbidity due to local invasive procedure. Engineered bone grafts, theoretically manufactured with BMPs, MSCs and scaffold combinations, may exhibit remarkable advantages in osteoconduction and osteoinduction as compared with the traditional therapeutic interventions and holds very promising future to aid bone fracture healing.

In 1965, Urist found that the active substance responsible for bone induction was a mixture of proteins, which afterwards were named as BMPs [1]. In the following

decades, BMP genes were cloned and the recombinant BMPs were shown to be biologically potent to induce bone regeneration [2]. Extensive studies have been performed in rodent cells and animal models, followed with the clinical use of recombinant BMPs in bone fracture healing, spine fusion, as well as dental tissue engineering [3-5]. Two products containing rhBMP-2 or rhBMP-7 in collagen-based carriers were approved by FDA in 2002 to use in spinal fusion and long bone non-unions [6,7]. Although numerous preclinical animal studies demonstrated that BMPs could promote bone repair *in vivo*, as evidenced by healing critical-sized bone defects, accelerating fracture repair, generating spinal fusions, and enhancing the mass of dental bones [8], the osteoinductive activity of BMPs reduced when study moves from rodent models to higher mammals [9]. The success rate for BMPs in human clinical studies is less than desirable [10]. It has been a longstanding question as to why BMPs are relatively ineffective in inducing osteogenesis in human, as compared with outcomes from animal models.

Several studies indicate that BMP antagonist, noggin, was induced by BMPs, and it subsequently impaired osteoinductive activity of BMPs in rat osteoblasts and mouse embryonic cells [11,12]. In our study, the effect of noggin on BMP signaling was investigated in human MSCs, the progenitors of osteoblasts. We found that noggin was induced by BMP-2 in a time- and dose-dependent manner in human MSCs. Surprisingly we found that noggin suppression impaired BMP-2-induced osteogenic differentiation of human MSCs, as evidenced by down-

regulated expression of osteogenic marker genes, decreased ALP activity and calcium deposits. These data suggest noggin may have a positive role in osteogenesis of human MSCs. Our finding is contrary to the reported observations in murine and rat cell lines that noggin has a negative role in bone formation, illustrated by either over-expressing [13,14] or silencing of noggin gene [15,16]. However, our data are in agreement with a previous study that administrating exogenous noggin in human MSC culture increased ALP, calcium deposition, as well as up-regulated expression of several osteogenic marker genes [17]. These results suggested there might be a species difference for the role of noggin in the osteogenesis process of MSCs, although the detailed mechanisms are unclear yet.

Investigations on the effect of noggin on osteogenesis by down-regulating noggin have been conducted in murine cell lines [15,16]. Compared with Wan's study [15], our present study differs in two aspects: 1) we performed these experiments in human MSCs instead of mouse cell lines (MC3T3-E1 preosteoblasts and primary osteoblasts from CD-1 mice). It is known that rodent cells and human cells exhibit different responsiveness towards BMP-2 so that conclusion drawn from rodent cell experiments is probably not applicable to human cells [9,18]; and 2) we used synthetic siRNA duplex instead of siRNA plasmid construct with the virus promoter. Synthetic siRNA is safer and easier to handle and thus is more clinically relevant. Compared with Takayama's work [16], our study has two advances: 1) we used human MSCs instead of mouse

myoblast cell line (C2C12) and ICR mice; and 2) we detected the effect of noggin suppression by studying endogenous noggin under the normal condition, whereas in Takayama's study, they over-expressed noggin in C2C12 [16]. Therefore, our current study is more clinically applicable, compared with those two previous studies.

It has been well known that macrophages accumulate at the bone fracture injured sites and they play crucial roles in removing necrotic tissues and defending bacterial infection. Macrophages have been extensively investigated in soft tissue repair process where they were reported to be alternatively activated and function as "repair macrophages" [19-21]. When the skin is injured, monocytes enter the injured sites from the bloodstream, and peak at about one day after the injury at the injured sites [22]. Monocytes differentiate into macrophages once they reach the injured sites. Differentiated macrophages orchestrating wound healing by secreting various factors, including growth factors which increase cellular proliferation, chemoattractants which attract more immune cells to the injured sites, and factors which inhibit tissue growth when repair process is completed [23].

In contrast to abundant studies on macrophages' roles in soft tissue repair, only a few studies addressed their roles in bone fracture healing. It has been reported that macrophages are present in large numbers at the early stage of bone healing but become fewer in the bone formation area at the late stage [24]. While in

delayed union and non-union fractures, immunological study indicates that CD11b-positive monocytes/macrophages are consistently distributed in the connective tissue stroma with perivascular enrichment as long as 4-25 months after the bone fracture [25]. These data indicate that macrophages might be associated with delayed union and non-union. In addition, local administration of semi-soluble aminated glucan, probably through activating macrophages, results in an immature hypertrophic callus of poor biomechanical properties [26]. Providing the direct evidence for this concept, our study showed that differentiated macrophages suppressed migration, metabolism, and BMP-2-induced osteogenic differentiation of MSCs. Therefore, persisted macrophage activation might be one of the reasons for delayed union or non-union.

4.2 FUTURE DIRECTIONS

Our study suggests that macrophages inhibited BMP-2-induced osteogenesis of human MSCs. Thus the logical consideration would be that inhibiting macrophage activation might enhance the effectiveness of BMP-2. A number of molecules have been identified to suppress macrophage activation, including bisphosphonates (clodronate, alendronate, etc) [27], IL-10 [28], IL-4 [29], etc. Whether suppressing macrophage activation by these molecules could increase bone regeneration remains to be determined.

Although the macrophage conditioned medium was observed to inhibit migration, metabolic activity, and BMP-2-induced osteogeneses in human

MSCs, the underlying molecular mechanisms are still unknown. It is worth to note that differentiated macrophages could secrete various products, including cytokines, growth factors, hormones, and inhibitors of enzymes, etc [30]. Further studies to investigate what factors are in the macrophage conditioned medium are warranted to understand the molecular mechanisms of our findings.

Our data showed that noggin suppression by siRNA decreased osteogenesis of human MSCs. Besides, the duration of noggin knockdown was 7 days, which would allow us to knock down noggin at different time frames to investigate the role of noggin in specific stage of osteogenesis.

4.3 CONCLUSION

In the present studies, we found that macrophage conditioned medium reduced migration, metabolic activity, and BMP-2-induced osteogenesis of human MSCs. Noggin was induced by BMP-2 in human MSCs culture in a dose- and time-dependent manner. Noggin suppression by siRNA decreased the BMP-2-induced osteogenic differentiation and the proliferation of human MSCs. Manipulation on macrophages and noggin may provide us with novel strategies to aid bone fracture healing.

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