University of Alberta

Preclinical Development of Basic Fibroblast Growth Factor as a Systemic Bone Anabolic Agent for Osteoporosis Therapy

by

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ABSTRACT

Growth Factors, which are endogenous proteins that act on a variety of cells, are a feasible option for enhancing bone regeneration and repair. To assess their potential for systemic therapy for osteoporosis, we determined the effect of growth factors basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) on rat bone marrow stromal cells (BMSC) using *in vitro* cell culture studies. Both bFGF and BMP-2 were stimulatory for bone formation under select culture conditions. Although BMP-2 effects were more lasting on BMSC, bFGF was more potent for bone formation. We investigated responsiveness of BMSC explanted from 7-month old normal and ovariectomized (OVX) rats that were intravenously treated with 25 μg/kg bFGF for 2 weeks. Systemic bFGF stimulated bone formation in OVX but not in normal rats after only 2 weeks of treatment. The ability of bFGF for osteogenic stimulation at low administration dose shows great promise for osteoporosis therapy.

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LIST OF ABBREVIATIONS

ACS	absorbable collagen sponge
ALP	alkaline phosphatase
bFGF	basic fibroblast growth factor
β-GP	β-glycerol phosphate
BMC	bone marrow cells
BMD	bone mineral density
BMP	bone morphogenetic proteins
BMP-2	bone morphogenetic protein-2
BMSC	bone marrow stromal cells
BP	bisphosphonate
BSP	bone sialoprotein II
CPM	counts per minute
CT	computed tomography
DEXA	dual energy x-ray absorptiometry
DMEM	Dulbecco's modified Eagle medium
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF	growth differentiation factor
GF	growth factors
HA-TCP	hydroxyapatite-tricalcium phosphate
HBSS	Hank's balanced salt solution

hPTH	human parathyroid hormone
IGF	insulin-like growth factors
IGFBP	IGF-I-binding proteins
IV	intravenous
OCN	osteocalcin
OM	osteogenic medium
OP-1	osteogenic protein-1
OPN	osteopontin
OVX	ovariectomized
PCR	polymerase chain reaction
PDGF	platelet-derived growth factors
PLGA	poly (lactic-co-glycolic acid)
p-NP	p-nitrophenol
p-NPP	p-nitrophenol phosphate
rhBMP-2	recombinant human BMP-2
RT-PCR	reverse transcription polymerase chain reaction
SC	subcutaneous
SD	standard deviation
TGF	transforming growth factor

SCOPE OF DISSERTATION

Bone is a highly specialized and dynamic tissue whose maintenance is a result of the coordinated processes of bone formation and resorption. An imbalance in this orchestrated process leads to the condition referred to as osteoporosis, which is the most significant bone disorder observed today. An overview of bone biology, bone homeostasis and osteoporosis, and treatment options is presented in the INTRODUCTION section.

Bone formation is a cellular process that is coordinated by various growth factors, which are endogenous proteins that stimulate cellular proliferation and differentiation. The various growth factors, their cellular effects and their role in bone remodelling is elaborated in CHAPTER I. The differences between endogenous utilization of growth factors and their exogenous application are also discussed. Given the role of growth factors in bone remodelling and regeneration, and the need to repair compromised bone, significant amount of work has been done on growth factor therapy. An update on the progress of growth factor delivery strategies has been presented. Local and systemic administration of growth factors for bone regeneration has been discussed separately. Critical observations from animal studies have been emphasized, with special attention to the drug delivery aspects of growth factor administration. The adverse effects of systemic growth factor administration and future considerations for local and systemic delivery of growth factors have also been discussed.

Growth factors hold great promise for systemic therapy of osteoporosis, especially basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2). The effects of bFGF and BMP-2 on bone marrow stromal cells (BMSC) *in vitro* in cell culture was determined in our study and are presented in CHAPTER II.

Different concentration of growth factors, exposure duration (continuous or intermittent) and the source of cells (young or adult rats) were analyzed. The culture conditions under which both bFGF and BMP-2 were stimulatory for bone formation were examined. The differences in the stimulation of osteogenic parameters in BMSC by both bFGF and BMP-2 were also evaluated.

From our initial studies it was evident that bFGF is more potent than BMP-2 and lesser quantities of bFGF is required to be delivered into the body, which would likely result in reduced adverse extra-skeletal effects. Therefore, we used very low dose bFGF for systemic therapy in normal and ovariectomized (OVX) rats, the results of which are described in CHAPTER III. This study is the first reported investigation wherein the responsiveness of the BMSC explanted from both normal and OVX rats, which were intravenously (IV) treated with a very low dose of bFGF for two weeks, are compared head-to-head. The three main foci of the study were (1) to characterize the cellular response of BMSC from normal and OVX rats subsequent to IV treatment with a low dose of bFGF, (2) to evaluate and compare the effect of bFGF on bone mass in the tibiae of normal and OVX rats, and (3) to assess the changes in gene expression in the explanted BMSC. The BMSC from the bFGF-treated rats and the saline-treated control rats were assessed for osteogenic markers such as specific alkaline phosphatase (ALP) activity and mineralization. Bone mineral density (BMD) of the tibiae of both the treated and the control rats was also determined by dual energy X-ray absorptiometry (DEXA) analyses. Additionally, we also assessed the effect of systemic bFGF treatment on the level of gene expression in BMSC culture for extracellular matrix proteins such as osteopontin (OPN), osteocalcin (OCN) and bone sialoprotein (BSP) with respect to that

of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Significant differences in the stimulation of osteogenic parameters in BMSC from normal and OVX rats, were observed in our study.

In all, the research work presented here serves as a pre-clinical assessment of the use of bFGF for systemic therapy of osteoporosis. This work also lays the foundation for numerous additional studies as outlined in the CONCLUSIONS AND FUTURE AVENUES chapter, which would further expand upon our knowledge in the field of systemic therapies for osteoporosis using bone anabolic agents.

INTRODUCTION

Bone

Bone is a highly specialized and dynamic tissue whose primary function is to form the skeletal system, provide mechanical support to soft tissues and facilitate muscular action. Bone is also responsible for housing the brain and the spinal cord; supporting haematopoiesis and maintaining calcium levels in the blood. Bone tissue is highly adaptive, mobilizes mineral resources to cater to the metabolic demand of the body, and is capable of healing itself completely. The three main cell types responsible for bone tissue maintenance are the osteoblasts, osteoclasts and osteocytes [1]. Osteoblasts, which are derived from pluripotent mesenchymal stem cells, are responsible for laying down the extracellular bone matrix. While osteoclasts, which are derived from the haematopoietic precursor cells located in the monocytic fraction of the bone marrow, resorb the bone matrix. Osteocytes, on the other hand, are highly specialized and fully differentiated osteoblasts, which account for 90% of all the cells in the adult skeleton [1].

Formation of bone

The formation and maintenance of bone is the result of complex, coordinated and synergistic interactions between the different bone cell types. During morphogenesis, the formation of bone occurs by one of the two developmental programs referred to as intramembranous and endochondral ossification. Certain parts of the skeleton including the cranial vault, some facial bones, and parts of the mandible and clavicle are directly formed by osteoblasts by intramembranous ossification, whereas the rest of the skeleton

is formed via a cartilage template, which later on gets replaced with bone by endochondral ossification [1].

a. Intramembranous ossification

During intramembranous ossification, wherein the skull and the other flat bones are formed, the mesenchymal cells condense and differentiate directly into osteoblasts. These osteoblasts secrete bone-matrix proteins, resulting in calcification and finally bone formation, without a cartilage intermediate [2].

b. Endochondral ossification

During endochondral ossification, the axial and appendicular portions of the skeleton are formed, involving a cartilage intermediate. As opposed to the intramembranous ossification, the cells in the endochondral skeletal elements differentiate into chondrocytes, which then form the cartilage template that later on is replaced by endochondral bones [3]. It is important to note that endochondral ossification is not only responsible for the formation of most of the skeletal bones, but also plays an important role in fracture repair and ectopic bone formation.

The molecular mechanisms that induce osteogenesis in the intramembranous skeletal elements are distinct from those operating during chondrogenesis in the endochondral skeletal elements. Molecular mechanisms involved in the formation of the endochondral skeletal elements are discussed below.

Molecular mechanisms of endochondral bone development

The process of endochondral bone formation can be divided into four sequential but overlapping phases: 1) mesenchymal cell recruitment and proliferation, 2) chondrogenesis, 3) osteogenesis, and 4) bone remodelling. Chondrogenesis is the process during which mesenchymal cells condense and differentiate into chondrocytes. These newly formed chondrocytes deposit an extracellular matrix that is cartilage-specific, and undergo unidirectional vectorial proliferation resulting in parallel columns of dividing cells. The chondrocytes after exiting the cell cycle undergo apoptosis, become hypertrophic (expressing predominantly type X collagen) and die. During osteogenesis which occurs simultaneously, some of the mesenchymal cells surrounding the cartilages differentiate into osteoblasts, and invade the zones of hypertrophic chondrocytes together with blood vessels and osteoclasts. While the osteoclasts degrade the hypertrophic cartilage matrix, the osteoblasts produce a bone-specific matrix using the degraded cartilage matrix as a scaffold. Finally, the formation of bone initiates the process of remodelling, which represents a delicate balance of two opposing processes, the formation of bone by osteoblasts and its resorption by osteoclasts [4].

Bone homeostasis

Bone being a highly dynamic organ, undergoes continuous regeneration, referred to as remodelling. The bone remodeling process begins with resorption of bone by the osteoclasts and is followed by formation of new bone by the osteoblasts. The delicate balance between bone formation and bone resorption is referred to as bone homeostasis, which is highly regulated [5]. Bone remodelling continues throughout the life of

vertebrates at spatially and temporally discrete skeletal sites thus ensuring the maintenance of bone homeostasis. Remodeling of bone is necessary not only for skeletal growth and development but also for the maintenance of the normal bone structure. During remodelling that takes place asynchronously in 'bone multicellular units' (BMUs) throughout the skeleton, old and compromised bone is continually replaced with new bone, in order to maintain bone strength and integrity [5]. *In vitro* studies on new bone formation showed that rat calvarial cells could make bone on bone slices with crevices and grooves excavated on them, suggesting that the topography of the bone affected the timing and extent of new bone formation. Further on, *in vivo* studies showed that new bone formation took place in the resorbed spaces vacated by osteoclasts, with the participating cells able to sense the spatial limits (probably through autocrine and paracrine chemical communication) [6]. Several studies strongly indicate the presence of different factors that contribute to the coupling of new bone formation to resorption.

Imbalance in bone homeostasis - Osteoporosis

Imbalance in bone homeostasis occurs when the processes of bone resorption and formation are not matched. A constant supply of osteoclasts and osteoblasts is essential for bone homeostasis. Any changes in their numbers and function would result in an imbalance between bone formation and resorption. An imbalance wherein the rate of resorption is higher than the rate of formation leads to irreversible bone loss and predisposition to fractures [5].

Osteoporosis, which is the most significant bone disorder observed today, is a consequence of such an imbalance. It is characterized by low bone mass and deterioration

of bone tissue leading to increased bone fragility and risk of fracture. Osteoporosis has been classified as type I or post-menopausal osteoporosis and type II or senile osteoporosis. In post-menopausal osteoporosis there is an accelerated trabecular bone resorption related to estrogen deficiency, and is characterized by fractures involving the spine and the wrist. This is mainly prevalent in post-menopausal women typically between ages 50 and 65 years. In senile osteoporosis there is a proportional loss of cortical and trabecular bone, and is characterized by fractures of the hip, the proximal humerus, the tibia and the pelvis, and is prevalent in elderly women and men. Senile osteoporosis is related to age-dependent decrease in bone formation, diminished adrenal function, reduced intestinal calcium absorption and secondary hyperparathyroidism [7]. In patients with osteoporosis, there is a decrease in mean wall thickness, presumably reflecting the inability to repair adequately the resorptive defects, made during normal osteoclastic resorption.

The epidemic proportions of osteoporosis become apparent from the estimate that 1.4 million Canadians suffer from it, with one in four women and one in eight men over the age of 50 being affected [8]. These alarming statistics put in perspective the need to effectively combat the disease, and also highlight the significance of developing newer therapeutic strategies.

Current treatment options for osteoporosis

The therapies used to treat osteoporosis can be classified into two broad categories referred to as anti-resorptive and bone anabolic therapies [9].

a. Anti-resorptive therapy

Anti-resorptive therapy depends exclusively on controlling osteoclasts, aiming for a net decrease in bone resorption. It can be defined as primarily anti-remodeling, since it slows down the rate of bone remodeling, eventually leading to less bone apposition than observed with the fundamental anabolic therapies [10]. Anti-resorptive agents combat the mechanisms causing bone loss by reducing the rate of bone remodeling and increasing the mineral content of the bone by producing shallower resorption cavities [9]. However, they do not restore bone strength by increasing the bone mass. A few of the currently used anti-resorptive agents are estrogen, raloxifene, and the bisphosphonates alendronate and risedronate [11]. Only moderate or minimal results could be expected from such a treatment, since this therapy does not replace the compromised trabecular connectivity. Anti-resorptive therapies are not able to generate a bone turnover that is higher than normal, wherein a bone turnover slightly higher than normal is necessary for the faster regain of bone loss [12].

b. Bone Anabolic therapy

Bone anabolic or formation therapy is aimed at increasing the extent of bone formation. Increasing bone formation appears to be a rational approach for the reversal of bone fragility, provided that the anabolic agent could increase bone mass and cortical thickness, trabecular thickness and connectivity, and also allow normal bone mineralization to occur [9]. Current bone anabolic therapies include the use of oral strontium ranelate and intermittent parathyroid hormone therapy [11].

Future therapeutic strategies for Osteoporosis

Since osteoporosis is a major health concern that affects hundreds of millions of people globally, effective treatment options are necessary to combat the disease successfully. Currently available treatment options have not been able to completely suffice the requirements of enhancing bone mass and reducing bone fragility in the treated individuals. This emphasizes the need for development of novel therapeutic strategies and treatment options to efficiently treat the disease.

Analyzing the currently used therapies for osteoporosis, it is apparent that an ideal therapy would be the one that drives the bone resorption-to-formation ratio in favor of bone formation. Such an intervention will not only allow the patient to regain the lost bone mass but also help with the maintenance of the bone gained. This would ideally achieve a bone turnover rate where the bone formation rate is 2–3 times higher than normal, because a lower than normal bone turnover rate would effectively lead to a slow bone apposition rate that is not compatible with fast recovery [12]. There is a clear need for developing novel bone anabolic agents that would substantially increase bone formation, especially in severe osteoporotic individuals who have already suffered substantial bone loss. Targeting the bone anabolic process with these agents would result in a higher bone mineral density stimulating a positive bone balance, and thereby enhancing the recovery prospects of osteoporotic individuals.

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CHAPTER I

GROWTH FACTOR DELIVERY FOR BONE TISSUE REPAIR[1]

[1] The contents of this chapter have been previously published in: Varkey M, Gittens SA and Uludağ H, Growth Factor Delivery for Bone Tissue Repair: An Update, *Expert Opinion on Drug Delivery* (2004) 1(1): 19-36.

3.1 Introduction

Growth Factors (GFs), also known as cytokines, are endogenous proteins that act on a wide variety of cells and direct their actions via cell-surface receptor binding and activation. The actions of GFs on cells are long lasting, so that a single exposure of cells to GFs results in long-term (weeks to months) cellular effects. The observed cellular effects are multifaceted, typically manifested as:

- an onset of vectorial migration (chemotactic effect)
- a stimulation of cell division (mitogenic effect)
- an induction of cellular differentiation (morphogenic effect)
- an initiation of programmed cell death (apoptotic effect)
- a modulation of metabolic activity
- a combination of the above

GFs are typically multimodal, exhibiting a different mechanism of action depending on the concentration and/or exposure time, as well as the phenotype of the target cell. Because developmental bone formation is an orchestrated cellular process tightly controlled by the actions of GFs, GF therapy is an obvious strategy when the bone integrity is compromised and bone tissue needs to be repaired. Such a strategy aims to enhance the local presence of bone-depositing osteoblasts, either by attracting the cells to the repair site or by inducing the proliferation of local undifferentiated, precursor cells, followed by the transformation of precursor cells into osteoblastic phenotype. Angiogenesis is an integral part of this process, as it provides the necessary nutritional support for the newly formed tissue, as well as providing a cell source for further remodeling of the tissue formed at the outset. There is no magic bullet when it comes to

the choice of the appropriate GF for bone repair. Mitogens such as platelet-derived growth factors (PDGF), morphogens such as bone morphogenetic proteins (BMPs), metabolic regulators such as insulin-like growth factors (IGFs), as well as angiogenic proteins such as basic fibroblast growth factor (bFGF), have all been used with some degree of success in bone repair in animal models.

Although GFs are a logical choice for enhancing bone repair, there is an important distinction between the endogenous utilization of GFs for bone induction and modeling, and the exogenous application of GFs for bone repair. The former typically involves local production of minute quantities of the proteins continually, whose activities are regulated by binding to extracellular matrices and presentation to the cells. Exogenous GFs, on the other hand, rely typically on the one-time application of supra-physiological concentrations of the proteins, whose local presence needs to be maintained throughout the repair process. When GFs are used for local bone repair, the critical drug delivery challenge is to identify a biomaterial carrier that maintains a sufficient concentration of the proteins at the application site for the duration of the repair process, while providing an appropriate support for the healing process. The latter requires the biomaterial carrier to provide an appropriate mechanical environment, to degrade at a tailored rate (to match the tissue formation) and to exhibit sufficient cell compatibility and porosity for induction of functional tissues. When GFs are used for systemic augmentation of bone tissue, the critical drug delivery challenge is to deliver a high dose of the proteins to bones throughout the body, with minimal distribution to extraskeletal sites. This requires the GFs to be 'bone-seeking', with an ability to exhibit preferentially an affinity to bones. Unlike local regeneration, systemic therapy is possible with multiple administration of

GFs. In both systemic and local therapy, stimulation of bone repair relies on the local presence of the exogenous proteins. That is, no further pharmacological activity is achieved once protein levels are reduced to below a threshold concentration. To circumvent this limitation, delivery of the GF genes, rather than the proteins, has been receiving much attention in recent years. The gene delivery approach allows local production of the proteins to modulate the healing process, and may better mimic the endogenous bone regeneration and healing process. The critical drug delivery challenge in this case is to design gene carriers that can effectively transfer a therapeutic gene into an endogenous system, either directly to the local cells *in vivo*, or indirectly to expanded cells *ex vivo* intended for administration into a repair site.

This chapter will summarize recent (2001 – 2004) progress in GF delivery for bone repair. The use of GFs has recently been summarized by the authors [1], and this chapter is intended to update the concepts explored in the previous review. The organization of this chapter is such that the GFs used in local and systemic bone regeneration are discussed separately. Critical observations from animal studies have been emphasized, with special attention on the drug delivery aspects of GF administration. Other researchers have also reviewed the GF therapy for bone tissue engineering [2-8], and the reader is encouraged to consult these reviews for an independent point of view.

3.2 Local delivery of growth factors

The potential of a GF needs to be evaluated in a non-human primate model before efficacy testing in clinical studies. It has been recognized that the threshold/optimum

dose for exogenous application of BMPs varies among species at different phylogenetic levels [9, 10], and non-human primate studies are appropriate to guide the dose selection in clinical studies. Among the GFs delivered in non-human primates are members of the transforming growth factor (TGF) family, such as TGF-β1 and TGF-β2, members of the BMP family, such as BMP-2, BMP-7 (also known as osteogenic protein-1, OP-1) and growth differentiation factor (GDF)-5 (also known as cartilage-derived morphogenetic protein-1), bFGF, PDGF and IGF-I [1]. As a result of multi-centre studies [11-14], recombinant human BMP (rhBMP)-2 has recently been approved for clinical intervention in acute tibia fractures treated with intramedullary nailing and in interbody fusion of the lumbar spine with specific types of spinal devices (LT-CAGE® lumbar tapered fusion device, and InterFIX™ and InterFIX RP™ threaded fusion devices [Medtronic, Inc.]). BMP-7, as a component of an implantable device, was approved as an alternative to autograft for long bone non-unions, in which the use of autograft is unfeasible and alternative treatments have failed, and in revision spinal fusions. In addition to the recombinant proteins, naturally-derived osteogenic 'cocktails' were shown to be effective in clinical studies [15], but their clinical application is likely to be hampered due to difficulties in quality control/assurance of the drug product (i.e., unlike relatively pure recombinant proteins, naturally isolated protein mixtures are more difficult to obtain with a consistent composition and a desired osteopotency).

With the approval of effective GF therapies for local bone healing, the challenge for the next-generation clinical devices will be an improvement in the current standard of care.

Improved therapies are desirable for the following reasons:

- to increase the assurance of healing, as existing therapies are < 100% effective in the approved clinical indications (e.g., rhBMP-2 application in spinal fusion reports failure rate of 5.6% after 24 months [14], rhBMP-2 in fracture healing require a > 20% secondary interventions [11], and OP-1 application in spinal fusion reports a success of 55 – 75% depending on the evaluation criteria [16]).
- to limit excessive bone formation sometimes seen in clinical studies [12] that may limit bone induction at the site of therapy.
- to widen the scope of application for bone sites not already approved
- to accelerate the healing process and restore the lost function as quickly as possible
- to improve delivery of the therapy, and, in particular, to establish minimally invasive approaches for device application, for example, by injectable delivery of the therapy as opposed to existing modes of implantable delivery

These improvements are likely to emerge by employing new GFs or enhancing the design of the current delivery systems and/or biomaterial carriers with which the osteogenic proteins are implanted.

Towards these goals, several non-human primate studies using rhBMP-2 and OP-1 have recently been reported (Table 1.1). The impetus for the primate studies with the GFs already approved for human use is multifaceted. First, a better understanding of regenerative events at specific anatomical sites, such as the changes in cellular events and carrier integrity, will lead to better understanding of osteoinductive device performance in clinics, especially when the devices are implanted at different sites. Given the well-

Table 1.1 Protein Growth Factors shown to be effective in non-human primate models of local bone regeneration

Protein	Delivery System	Model	Critical observation(s)	Ref
rhBMP-2	PLGA matrix containing rhBMP-2	Mandibular osteotomy defect	Co-delivering bone marrow cells along with rhBMP-2 / PLGA matrix greatly enhanced the extent of bone formation. Bone marrow cell alone was also as effective, but not rhBMP-2/PLGA alone. Small sample size in treatment groups hampers conclusive results‡.	[17]
rhBMP-2	Collagen sponge	Mandibular and cleft osteotomy in *Macaca fascicularis*	rhBMP-2-induced new bone was subsequently able to 'accept' functional titanium implants. Aged animals (~ 20 years) responded to rhBMP-2 similarly to that of younger animals (6 – 9 years). rhBMP-2 induced bone formation more effectively than autogenous bone graft in a cleft osteotomy.	[18] *
rhBMP-2	Gelatin/PLGA sponge	Alveolar mandible defect in Rhesus monkeys	2.8 mg rhBMP-2 yielded radiographic evidence of bone formation initially at 4 weeks with full effect at 12 weeks, unlike no bone formation in control (no implant) group. The deposited bone underwent maturation in both lamellar and trabecular spaces between 6 and 12 weeks. Significant variation in induced bone was noted among the animals.	[19]
rhBMP-2	Gelatin/PLGA sponge	Mandibular osteotomy defect (30 mm) in Rhesus monkeys	9 mg of rhBMP-2 gave sufficient bone for implant placement in 20 weeks, where radiographically new bone was indistinguishable from the original bone structure. Histological analysis indicated rapid, unorganized (woven) bone deposition initially, followed by remodeling after 15 weeks. The high dose of implanted was noted, but no attempts were made to explore the necessary threshold dose for osteoinduction.	[20]
rhBMP-2	Calcium/phosph -ate cement	Fibula osteotomy defect in baboon	An injectable calcium/phosphate cement (α-BSM™) facilitated bony bridging at > 0.35 mg/ml rhBMP-2 concentration (8 weeks), unlike the surgical control (no intervention) and the injection of the cement alone (0 mg/ml rhBMP-2).	[21]
rhBMP-2	ACS embedded with HA-TCP particles	Bilateral Postero-lateral inter-transverse process arthrodesis in Rhesus monkeys	HA-TCP (5:95 and 15:85 ratios) embedded in an ACS gave easier radiographic monitoring of bone formation, faster resorption of carrier, and better histological bone formation in the centre of fusion mass, as compared with 60:40 HA-TCP embedded ACS. The authors state that "failed osteoinduction results in rabbits have a high predictive value for failure in primates, (and) successful rabbit results do not ensure success in primates". Growth factor concentration, rather than the dose, was proposed as the critical parameter for the implant success.	[22]
rhBMP-2	ACS embedded with HA-TCP or allograft bone particles	Bilateral Postero-lateral inter-transverse arthrodesis in Rhesus monkeys	Successful fusion (L4-L5) with only one of three autogenous bone grafts, whereas six of six rhBMP-2 / ACS implants augmented with ceramic particles/bone chips gave successful fusion. There was no ectopic bone with both particulate-embedded sponges, which gave an equivalent response at rhBMP-2 doses (3 mg) that was lower than the historical results in this animal model.	[23]

rhBMP-2	ACS	Alveolar osteotomy defects in cyno-molgus monkeys	A relatively low dose of rhBMP-2 implantation with ACS (0.1 mg/defect) did not result in significant benefit in new bone induction and osseointegration as compared with control (no rhBMP-2 treatment), indicating significant regenerative capability of the alveolar site.	[24]
rhBMP-2	ACS	Osteotomy defect in cranium of Rhesus monkeys	0.1 mg rhBMP-2/site induced 71% bone in treated defects, whereas controls (ACS alone) gave 28% bone in defects. The rhBMP-2 receiving animals exhibited good osseointegration and no apparent adverse effects on animals.	[25]
OP-1 and rhBMP-2	Bovine demineralized bone matrix	Mandibular osteotomy defect in baboons	OP-1 in the utilized model provided greater extent of cementogenesis, but reduced alveolar bone formation as compared with rhBMP-2 application. Simultaneous application of the GFs did not synergistically enhance periodontal regeneration§.	[26]
OP-1	Porous HA matrix	Calvarial osteotomy defects in baboons	Whereas 100 μg OP-1/site was effective for bone deposition, 500 μg OP-1/site gave a more robust, but occlusive, bone formation pattern at the implant periphery after 30 days. Longer times resulted in complete remodeling of the induced bone, filling the pores of implants. HA from different sources exhibited some, but not overwhelming, differences in the extent of new bone induction. A faster resorbing carrier was needed in this model.	[27]
OP-1	Bovine bone derived collagen	Ulnar (2 cm) osteotomy defect in African green monkeys	1 mg OP-1 induces new radiographic bone at 6 – 8 weeks post-surgery. DEXA analysis indicated robust mineralization at 12 weeks. Increased cellular proliferation was evident as early as 1 week and throughout the 20-week healing period and, consistent with DEXA, significant bone deposition was evident histologically at 12 weeks onwards.	[28]
bFGF	Gelatin	Mandibular osteotomy defect in baboon	bFGF increased new bone formation as a function of implant dose. Induction of new cementum was also increased with bFGF application, and no undesired activities (e.g., ankylosis or root resorption) were noted.	[29]
bFGF	Injectable gelatin sponge (open tibia injection)	Ulnar osteotomy defect in Cyno-molgus monkeys	Complete healing of osteotomy defect in 10 out of 10 animals at 10-weeks with 200 μg/site bFGF, but only 6 out of 10 in control (no bFGF) group. Beneficial effect of bFGF on bone mineral density and mechanical properties.	[30]
TGF-β_3	β-TCP matrix	Partial defect model in vertebral baboon (L2-L4)	Unlike sham operation (no implants), β-TCP implants with and without TGF-β_3 (50 mg/ml) were osseo-integrated. TGF-β_3 appeared to accelerate resorption of implants at their periphery and caused deposition of well organized cancellous bone at the bone–implant interface.	[31]

*Summary of previously published studies. ‡Authors' conclusions. §Note that the BMP-2 used in this study was derived from an *Escherichia coli* expression system, unlike the Chinese hamster ovary-derived BMP-2 used in other studies. ACS: Absorbable collagen sponge; bFGF: Basic fibroblast growth factor; DEXA: Dual Energy X-ray absorptiometry; HA-TCP: Hydroxyapatite-tricalcium phosphate (15:85%); OP: Osteogenic protein; PLGA: Poly (lactic-co-glycolic acid); rhBMP: Recombinant human bone morphogenic protein; TGF: Transforming growth factor.

reported histological results, the time course of cell infiltration, osteogenic differentiation, initial bone deposition and long-term remodeling are beginning to be better understood [27, 28, 31]. Such well-controlled cellular studies, although possible [10] are difficult to conduct in clinical studies. Primate studies have also explored critical device properties that contribute to better performance, such as the exploration of an appropriate therapeutic dose or improvement of mechanical properties of existing implants. Only primate studies can mimic the clinical situation, especially for the unique mechanical environment present in the spinal fusion indication [23]. Augmentation of existing devices with bone marrow cells has been attempted with promising results, as this procedure incorporates cellular elements that are absent in existing devices [17]. In addition to cellular effects, it is likely that the presence of other cytokines, such as bFGF, may contribute to the beneficial effect of bone marrow. Finally, new biomaterial carriers are continually explored to overcome certain limitations of the existing clinical delivery systems [21]. The biomaterials are generally regarded to act as 'depots' of the delivered growth factors, maintaining a high enough concentration of the proteins during formation of new mineralized tissue. Whether this is by direct binding or simply by acting as a physical barrier to prevent free diffusion of the proteins is open for debate [32]. The latter is likely to be the case, given the wide array of biomaterials used for successful bone induction: collagen, gelatin, PLGA, various hydroxyapatites (HAs) and tricalcium phosphate (TCP) (Table 1.1). One critical limitation of the existing clinical delivery systems, absorbable collagen sponge in the case of rhBMP-2 and demineralized bone matrix from bovine in the case of OP-1, is the lack of mechanical strength, limiting the therapy to non-compressive environments or utilization of protective cages in

compressive environments. Mechanically-resilient HA [26] and TCP [31] capable of effectively allowing bone regeneration are welcome additions to the repertoire of biomaterials in this aspect. It is also possible to impregnate the collagen sponges with particulate biomaterials to enhance its compressive strength [22, 23]. A granular HA-TCP composite has already been tested in a pilot clinical study, in which 20/20 patients receiving rhBMP-2 exhibited successful posterolateral spine fusion based on radiographic assessment [33].The mineral-based biomaterials are known to bind BMPs avidly [32, 26], but direct demonstration of this has not been attempted in primates, only in smaller animal models. With mechanically-resilient carriers, biomaterial degradation is a critical challenge, as they can, in theory, impede mineralized bone deposition, unlike the malleable collagenous implants. Combination biomaterials, such as the gelatin sponges coated with polymeric PLGA for increased durability may offer a compromise between faster degradation (as compared with mineral-based carriers) and better space creation (as compared with collagenous carriers).

Apart from rhBMP-2 and OP-1, the only other GFs utilized in non-human primates were bFGF (a potent angiogenic factor and a mitogenic protein especially on preosteoblast cells) and TGF-β3 (a regulator of cell proliferation and differentiation). Whereas the beneficial effect of bFGF was evident in the utilized model [30], the beneficial effect of TGF-β3, despite clear evidence of unique microscopic changes at the implant site, was not obvious [31]. No dose-response studies were reported for TGF-β3 and it is likely that a higher dose might have revealed a more robust response in this animal model (rhBMP-2 concentrations used in spinal fusion is > 1 mg/ml, whereas the TGF-β3 dose was 0.05 mg/ml in this study). The bFGF dose in the primate ulnar

osteotomy model (~ 200 μg/site) appeared to be lower than the BMP-7 dose used in a previous study (~ 1 mg), although direct comparison of the two studies is difficult given the differences in the implanted biomaterial carriers (gelatin versus bovine demineralized bone matrix, respectively) and the created surgical defects (unspecified thinner osteotomy versus 2 cm defect, respectively). Preliminary studies have also indicated this protein to be potent in inducing osteogenic activity. For example, enhanced mineralization in bone marrow cells as compared with BMP-2 (Figure 1-1). Nevertheless, proteins more potent than the available BMPs may lead to more cost-effective therapies with reduced chance of side effects, as a lesser quantity of the exogenous protein needs to be administered. Now that the BMPs have become the standard therapy, it will be imperative to compare the potency of bFGF (and other GFs) head-to-head with BMPs, so that the true potential of the emerging GFs can truly be assessed.

It is also noteworthy that no primate studies have been reported with GDF-5, PDGF and IGF-I, since the compilation of the previous review [1]. A combination therapy, in which multiple GFs are delivered simultaneously to a defect site, was reported in baboons using rhBMP-2 and OP-1 coadministration, but the two GFs did not act synergistically in the chosen animal model. Each protein instead led to a unique morphological transformation at the utilized mandibular osteotomy site [27] (see Table 1.1). Several studies demonstrated synergistic effects of GF combinations in lower animals, including: (i) FGF-4 and BMP-2 in the rat ectopic model [34]; and (ii) IGF-I and TGF-β in a rat fracture [35] and a sheep cervical spine fusion model [36], in which a GF dose (150 μg IGF-I and 30 μg TGF-β1) gave the optimal response [37]. An early time point in the sheep cervical spine fusion model showed that the bone formation obtained

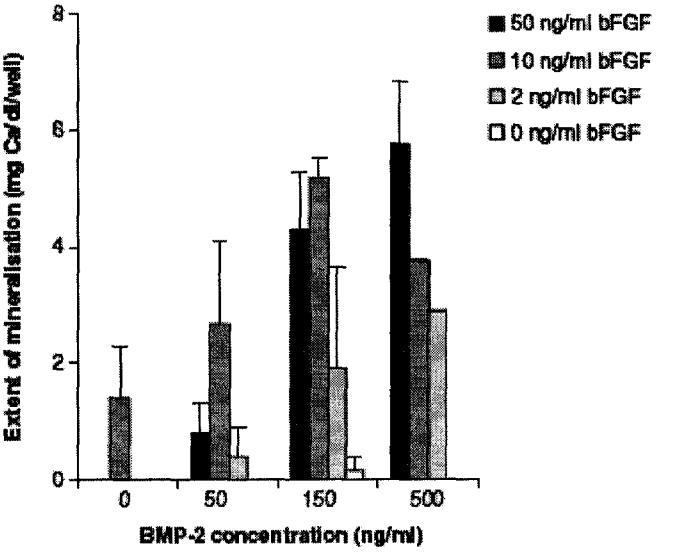

Figure 1-1. Effects of bFGF and BMP-2 treatment on mineralization of bone marrow stromal cells (BMSC). BMSC were harvested from ~32 week-old female Sprague-Dawley rats and cultured as described in Haque et al. 2004. The cells were treated for 1 week with a basal medium (DMEM + 10% fetal bovine serum) containing the indicated GF combinations (0, 50, 150 and 500 ng/mL BMP-2, each BMP-2 concentration supplemented with 0, 2, 10 and 50 ng/mL bFGF). The BMSC were then incubated in an osteogenic medium (DMEM + 10% fetal bovine serum medium supplemented with 3 mM ß-glycerolphosphate + $3x10^{-8}$ M dexamethosone) without any other GFs and the extent of mineralization was assessed with a colorimetric calcium assay. Note that; (1) there was increased mineralization as the concentration of BMP-2 was increased, and; (2) bFGF at very low doses (2-10 ng/mL) acted synergistically with BMP-2 for an enhanced mineralization. Even in the absence of BMP-2 (i.e., 0 ng/mL concentration), bFGF was capable of stimulating mineralization at 10 ng/mL concentration and the highest concentration of BMP-2 (500 ng/mL) required bFGF supplementation for mineralization after 1 week.

with dosing 150 μg IGF-I and 30 μg TGF-β1 was equivalent to that obtained by using comparable concentrations of BMP-2 (150 μg), suggesting that other GFs might substitute for the BMPs already approved for clinical application. Studies in primate models would be required to validate these results before these approaches progress to the clinics [36].

Finally, progress towards minimally-invasive delivery of GFs is being made by developing injectable biomaterial carriers that retain the proteins at an application site without significant protein loss over the course of the healing period. The bFGF study of Kawahuchi et al. was conducted with an injectable formulation of GF and gelatin, except it was injected in an open defect site, rather than percutanously [30]. A calcium / phosphate-based biomaterial percutanously injected into an osteotomy defect was shown to be effective in delivering rhBMP-2 in a baboon fibula model [21]. The effective concentration of rhBMP-2 was > 0.35 mg/ml, but no information about the injection volume was reported, so that its comparison with effective bFGF concentration could not be performed. Both gelatin and calcium/phosphate are degradable, so that they are not likely to impede new tissue induction in situ on injection. It is not clear whether the formulations used have been optimized, as it is possible to engineer the physicochemical properties of biomaterials and modulate the *in situ* retention of GFs, as was demonstrated with synthetic polymers co-injected with rhBMP-2 [38]. Efforts to engineer naturally-occurring biomaterials (such as collagen and its denatured derivative gelatin) to 'solidify' on contact with physiological milieu and sequester the injected proteins will greatly facilitate the regenerative therapies not possible with existing approaches, for example, in closed fractures and in local degeneration such as osteonecrosis.

3.3 Systemic delivery of growth factors

Based on the beneficial effect of GFs in local bone regeneration, systemic administration of GFs has been pursued for the augmentation of bone tissue throughout the skeletal system. Since the previous review, human parathyroid hormone (hPTH) 1-34, has been approved for osteoporosis therapy (see [39] for a review). Other GFs are continued to be explored for this indication and a summary of recent preclinical studies have been provided in Table 1.2. Unlike the local delivery, these studies have been conducted without a delivery system that targets the proteins specifically to bone. In this case, the proteins distribute throughout the body to establish a certain concentration in each organ, and it is expected that: (i) a beneficial pharmacological activity arises at bone sites due to the attainment of a therapeutic concentration; and (ii) no undesired activities are induced at extraskeletal sites due to an inability to reach to a pharmacological concentration at these sites. Most of the studies explored the beneficial effect of bFGF in the female ovariectomized (OVX) rat model, which is an established animal model for type I osteoporosis.

The well-established anabolic effect of IGF-I is still being explored, even though clinical studies involving long-term IGF-I treatment in postmenopausal women whose circulating IGF-I levels were elevated to levels observed in young women confirmed its ineffectiveness in increasing bone mineral density (BMD) or strength [51]. Preclinical studies, however, are yielding additional information about the effect of delivery mode on the obtained anabolic effect. In an IGF-I-deficient MIDI mouse model, IGF-I was shown to be more effective in producing significant increases in total BMD and in femoral BMD

Table 1.2 Systemic administration of growth factors in a preclinical model of postmenopausal osteoporotic (ovariectomized) rodents

Protein	Route	Dose and Schedule of Administration	Observations	Ref.
bFGF	IV	Daily injections of 200 μg/kg for 14 days	An increase in osteoblast and osteoid surfaces at hematopoietic sites while an attenuation of growth factor effect at fatty marrow sites was observed.	[40]
bFGF + PTH	IV	Daily injections of 200 μg/kg bFGF for 14 days, followed by 5 days/week injections of PTH at 80 μg/kg	Tibial cancellous bone volume was found to be increased by 15% by the sequential treatment of bFGF and PTH. bFGF alone increased osteoid volume, osteoblast surface, and osteoid surface, but not the cancellous bone volume.	[41]
bFGF	SC	Daily injections of 1 mg/kg for 21 days	A 4-fold increase in osteoblasts surface and an 8-fold increase in osteoid surface was observed.	[42]
bFGF	IV	Daily injections of 200 μg/kg for 14 days	An increase in cancellous bone volume was observed. Additionally bFGF observed to up regulate gene expression for bone matrix proteins and IGF-I.	[43]
bFGF ± estradiol and bFGF ± hPTH	IV	Daily injections of 200 μg/kg bFGF for 15 days. A week after bFGF treatment, 3X/week injections 10 and 80 μg/kg estradiol for 4 weeks.	bFGF treatment showed evidence of new trabeculae that formed osteoid bridges with pre-existing trabeculae. The trabecular connections so established could be maintained with either estrogen or hPTH	[44]
bFGF ± hPTH	SC	Injections of 1 mg/kg bFGF (5 days/week) for 60 days	bFGF was observed to increase trabecular bone mass by increasing trabecular number and connectivity, while hPTH increased the trabecular bone mass by thickening existing trabeculae	[45]
bFGF ± estrogen	SC	Daily injections of 1 mg/kg/d of bFGF and 10 μg/kg estrogen for 3 weeks (4 days/week)	Increase in osteoid volume, osteoblasts surface and osteoid surface was observed with bFGF treatment. Bone anabolic effect of bFGF was not enhanced by estrogen combination therapy	[46]
bFGF	IV	Daily injections of 200 μg/kg for 7 or 10 days	Bone anabolic effects of the growth factor were observed to start as early as 24h post-injection of bFGF and increase with time	[47]
BMP-2	IP			[48]
IGFBP-5	SC	Daily injections of 30 μg/mouse for 8 weeks	Enhancement in bone formation and bone accretion was observed which were attributed to stimulation of osteoblast activity.	[49]
IGF-I	IP	Daily injections of 1.21 mg/g for 6, 11 or 16 days	Femoral length and volume and the number of osteoclasts was found to be significantly greater after IGF-I injection	[50]

when administered three times a day, as opposed to once-daily administration [52]. Due to the short half-life of IGF-I and its rapid clearance by the kidney, a single administration may not sustain free IGF-I levels sufficient to produce the desired pharmacological effects. Studies in mice have revealed the importance of IGF-I in regulating the number of osteoblast progenitors in bone marrow cells. A dose- and age dependent effect of IGF-I on trabecular bone formation rate has been observed in OVX rats [53]. Investigations into time dependent effects of daily dosing of IGF-I on the linear growth of femur in newborn mice revealed that trabecular bone volume as well as femoral growth increased with the duration of the therapy [50]. Previous experience indicated a beneficial effect of IGF-I-binding proteins (IGFBPs) when co-delivered with IGF-I, but a recent study indicated that IGFBP-5 administration alone was capable of stimulating bone deposition, presumably due to its stimulatory effect on osteoblastic activity [49].

Most recent studies have focused on the anabolic effect of bFGF. As before [1], intravenous injection of bFGF was beneficial in stimulating bone deposition, but this was recently extended to the subcutaneous route as well. This is a more acceptable delivery route for clinical application, but a much higher dose of the GF was needed for this route: 1 mg/kg [42] versus 0.2 mg/kg required for the intravenous route [40, 41]. The bFGF therapy was most effective at haematopoietic marrow sites, such as the lumbar spine [40], possibly due to differences in the cellularity between this type of marrow and the fatty marrow. The beneficial effect of bFGF was believed to be initiated after the withdrawal of the treatment, but Power et al. [47] reported that the increase in histomorphic indices of bone formation began as early as 24 h after initial treatment and was observed to

increase with time. The rapid anabolic effects of bFGF have been suggested to be due to conversion of bone-lining cells to osteoblasts, which would thereby augment the osteoprogenitor cell population [47]. Apart from its rapid skeletal effects, bFGF was capable of forming new bone within the bone marrow, as well as establishing osteoid connections between disconnected trabeculae [46]. The trabeculae developed by bFGF therapy formed osteoid bridges with the lattice, suggesting its ability to stimulate new trabeculae [44]. bFGF was observed to increase trabecular bone mass by increasing the trabecular number and connectivity, as opposed to hPTH (1-34), which acts by thickening the trabecular walls [45]. This finding opens up the possibility of using sequential treatment paradigms involving bFGF and hPTH (1-34) for the treatment of severe osteoporosis.

Successful application of BMPs for systemic therapy has recently been reported. Turgeman et al. reported that rhBMP-2 at 0.5 – 5 μg/mouse i.p. daily was capable of stimulating osteoprogenitor cell proliferation in bone marrow, based on colony formation assays in vitro, as well as increase in trabecular bone volume in type I and II osteoporosis models [48]. The bone induction was not consistently dose dependent, but a select group of animals exhibited sufficient bone induction to restore the lost bone density in some of the utilized models, but not all. Although hPTH was more effective in enhancing the BMD under similar conditions, the feasibility of utilizing BMPs systemically is an exciting prospect for systemic augmentation of skeletal integrity.

3.4 Adverse effects of systemic growth factor administration

Studies regarding acute toxicity from a single intravenous dose, or sub-chronic toxicity testing with a daily intravenous dose, of rhBMP-2 for 28 days, yielded negative results in rat and dog models [48, 54]. The lack of systemic toxicity could presumably be attributed to rapid clearance of rhBMP-2, as observed in pharmacokinetic studies in rat and non-human primate models: $t_{1/2}$ = 16 and 6.7 min in rats and nonhuman primates, respectively [54]. Similar to rhBMP-2, no significant physiological disturbances have been reported in rats and non-human primates following intravenous delivery of OP-1 [54]. No harmful renal effects of OP-1 have been shown. Rather, it has been observed that its intravenous administration prevents the loss of renal function associated with ischaemic rat injury [55]. In mice, intraperitoneally injected OP-1 did not give any ectopic bone induction at the injection site, and 30, 100 and 300 μg/kg OP-1 doses injected for 8 – 12 weeks did not lead to significant histopathological changes in major organs [56]. An antifibrotic effect of OP-1 in renal tissues was suggested as a possible mechanism for renal protection, but such effects of OP-1, if any, on bone tissue, have not been reported in that study. bFGF administration, however, has been recognized to cause significant undesired activities at extraskeletal sites. bFGF acts as a pleiotropic mitogen that influences the growth, differentiation and survival of a variety of cell types [57]. Adverse effects associated with intravenous treatment of rats with bFGF include anaemia, kidney and lung hypertrophy with extramedullary haematopoiesis, defective renal function [46], impaired bone mineralization [42] and retarded weight gain [46]. Subcutaneous bFGF treatment in rats was found to induce substantial reductions in haematocrit levels and a similar impairment in bone mineralization as observed during

intravenous treatment [46]. This impairment in mineralization has been found to resume during the bFGF withdrawal period, resulting in the calcification of the abundant osteoid formed post-bFGF treatment, and its conversion to bone [58, 47]. Although the mechanism of this defective mineralization at doses efficacious in stimulating systemic skeletal anabolic effects is not clear, the hypophosphatemia observed in OVX rats is thought to play a role [58]. As in the case of bone mineralization, the haematocrit levels also are found to return to normal after withdrawal of the GF treatment [47, 58].

3.5 Expert opinion: future considerations in growth factor delivery

3.5.1 Local delivery

Now that the first-generation GFs have become available for clinical bone repair, the research community is expected to devote significant resources to new and improved approaches for bone repair. Based on the published literature, it is not clear whether an obvious GF candidate exists that can replace the already approved GFs for local bone repair, namely the rhBMP-2 and OP-1. Several other GFs, such as the bFGF and TGF-βs, seem to be capable of initiating an effective repair process equivalent to the existing GF/biomaterial combinations, although head-to-head comparisons, especially in primate models, are sparse. Most primate studies are 'proof-of-principle' studies only, as the limited number of animal subjects utilized (typically less than four/treatment group) does not allow a thorough statistical evaluation of the outcome measures.

Improvements in GF formulations, however, are more likely to yield significant advances over the existing therapeutics. Although minimally invasive approaches will

enable convenient application of existing therapeutics, they are also likely to expand the scope of clinical bone repair. Injectable delivery of GFs in simple buffers is preferable in a clinical setting from a regulatory perspective, as complications, if any, related to biomaterial development are eliminated in this way. Although simple buffer injections could be effective for some GFs in small animal models, in which the healing period is relatively short (for example, rhBMP-2 in a rat fracture model [59]), co-injection with biomaterials is likely to be the reality in a clinical setting, in which the healing period will be significantly longer. Biopolymers such as gelatin, which has been successfully used in primate models, could be engineered to deliver some GFs (e.g., bFGF and TGF-β), but not others (e.g., BMP-2 and VEGF) in an injectable format in rodents [60]. A good correlation between the biopolymer residence time and the protein residence times suggested the feasibility of controlling the GF delivery rate with the co-injected carrier, again supporting the need to engineer the biomaterial carriers to control *in situ* levels of the proteins. This goal was also recently pursued with a synthetic terpolymer, poly-D,L-lactic acid-*p*-dioxanone-poly ethylene glycol, whose degradation rate was controlled to induce orthotopic repair in rats [61]. In addition to protein retention that determines local concentration of the proteins [32], recent studies of Maeda *et al.* also indicated the need for gradual release of the proteins *in situ* [62]. Using several collagen 'minipellet' formulations, these investigators noted the importance of both the initial burst and the subsequent sustained release in the extent of *de novo* bone formed subcutaneously [62]. Given an equivalent implant dose, fast-releasing formulations were found to result in better bone formation. Equally important, simple additives such as amino acids, glycerol and sucrose, were effective in modulating the BMP-2 interactions with collagen, and

controlling the *in vivo* release rate. Such biomolecules are more readily acceptable for clinical formulations as compared with new biomaterials whose safety profile requires a more thorough assessment. Formulating the GF delivery systems in a minipellet format might also be advantageous over the one-piece scaffolds, such as collagen sponges, which might restrict cell migration especially to the central region of an implant.

In contrast to diffusible delivery systems, GFs immobilized in proteolytically-sensitive scaffolds could provide an 'on demand-release-system' whose GF content is released *in situ* as a result of the proteolytic activity of extracellular proteins and cells [63]. Using a rat calvarial osteotomy model, matrix metalloprotease-sensitive synthetic hydrogels were shown to be supportive of BMP-2-induced bone formation [63]. The animal model utilized in this study was a rodent model, which gave a robust bone induction even with a collagen/rhBMP-2 implant of equivalent rhBMP-2 dose. It is likely that the beneficial effect of such a controlled release system is more readily manifested in larger animal models, in which implantation dose and burst release become more significant as the bone healing process is prolonged.

3.5.2 Systemic delivery

It is likely that no new GF will be available for systemic therapy in the near future, after the approval of hPTH (a possible exception might be novel analogues of hPTH with improved therapeutic index). A critical need in systemic therapy is the drug delivery systems that specifically target the systemically administered proteins to bones. Towards this goal, model proteins (albumin and lysozyme) were derivatized with bone seeking bisphosphonates, and the bisphosphonate–protein conjugates were found to give

up to seven fold increased bone deposition of the proteins depending on the bone site, post injection time and the route of administration [64]. The protein-targeting efficiency, defined as the ratio of 'BP–protein delivery to a bone site' to 'delivery of native proteins to the same site', was increased as a function of post-injection time after a single administration of the proteins in rats [64]. This was indicative of not only better initial targeting, but also better retention of BP conjugates at bone sites after the initial protein clearance. The imparted bone affinity was dependent on the conjugation chemistry [65], and the tether length between a protein and bisphosphonate [66]. Approaches to link multiple BP to a single protein attachment site also yield bone-seeking proteins with a lesser degree of modification [67]. Collectively, a foundation for targeting proteins to bone is being established, and efforts to apply this bone-targeting approach to GF delivery to bone are underway. The critical issue in this respect is the need to modify the GFs chemically, without losing their pharmacological activity. Modifying proteins without a loss of activity is feasible, such as biotinylation of bFGF and BMP-2 [1], but whether the same observation holds true for bisphosphonate modification remains to be seen.

In the absence of a bone-specific drug delivery approach, the clinical utility of GFs for systemic therapy will rely on minimizing their undesired activities at extraskeletal sites. The extraskeletal rhBMPs (rhBMP-2 and -7 in particular) appear to be tolerated to some extent in rodent models, but more thorough studies will be required in larger animals to evaluate their probable side effects. The undesirable effects of GFs were generally reversible after the cessation of GF therapy, so that a good control of the administration dose might allow one to strike the right balance between an effective

stimulation of bone formation at skeletal sites and undesirable activities at extraskeletal sites. It is likely that the local concentration of a GF is the primary determinant of the obtained pharmacological activities. If so, pharmacokinetics and biodistribution studies can shed light on GF concentrations in various organs as a function of the dose and frequency of the administered therapeutics. Instead of the efficacy studies, it will be more productive to determine the skeletal/non-skeletal ratio of the GFs first, and then utilize the optimal administration regimen (i.e., where skeletal/non-skeletal delivery of GFs is maximized) for the efficacy studies. The potential of GF candidates for systemic therapy is more realistically assessed in this way.

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CHAPTER II

IN VITRO OSTEOGENIC RESPONSE OF RAT BONE MARROW CELLS TO bFGF AND BMP-2 TREATMENTS[2]

[2] The contents of this chapter have been previously published in: Varkey M, Kucharski C, Haque T, Sebald W and Uludağ H. *In vitro* osteogenic response of rat bone marrow cells to bFGF and BMP-2 treatments. *Clinical Orthopaedics and Related Research* (2006) 443: 113-123.

4.1 Introduction

Protein growth factors are endogenous regulators of cells and several are responsible for mineralized tissue mass and are being explored therapeutically for bone tissue engineering. These growth factors have been used in clinics by implantation with biomaterial carriers for bone repair at a site of administration [30, 14]. The growth factors also have been administered systemically in preclinical models to stimulate bone deposition throughout the skeletal tissues [28, 19]. The growth factors particularly are attractive for systemic stimulation of bone formation in patients with osteoporosis. Unlike the osteoporosis drug bisphosphonates that inhibit osteoclastic activity, growth factors have the potential to directly enhance skeletal integrity by stimulating deposition of new mineralized tissue by bone marrow cells (BMC). Basic fibroblast growth factor (bFGF) and bone morphogenetic protein-2 (BMP-2) are two growth factors that act as a prototypical mitogen and morphogen, respectively [5]. The bFGF administered by intravenous route was shown to stimulate endocortical bone deposition in young (growing) [21] and ovariectomized rats [22, 36, 12] the latter serving as a model of postmenopausal osteoporosis. Angiogenic and mitogenic effects of the bFGF have been attributed for the beneficial effects at bone sites [21, 22, 36, 12]. Systemic administration of BMP-2 was attempted in two osteoporotic mouse models, which led to increased trabecular bone volume at femurs [34].

Human-derived BMC are sensitive to bFGF and BMP-2 treatments, but the reported osteogenic effects of these growth factors are conflicting. Lecanda et al [13] reported a BMP-2 dependent matrix mineralization parallel with enhanced alkaline phosphatase (ALP) activity and increased deposition of the extracellular matrix proteins

collagen-(I), osteopontin, and decorin. Frank et al [3] used a medium containing bFGF/dexamethasone combination and reported differences in some osteogenic markers (BMP-2, bone sialoprotein, and osteopontin expression), but not in others (ALP activity, collagen-(I) expression, and mineralization); bFGF treatment reduced colony formation and ALP activity of the BMC [16], but acted as a mitogen once successful BMC colonies were established [16, 15, 24, 35]. Clonal differences in the osteogenic effect of bFGF on human BMC [3] and BMC derived from a murine model [6]. bFGF alone (without dexamethasone) was incapable of stimulating osteogenic markers in one study [15], but increased ALP positive colonies in another [35]. The BMC in these studies were obtained from individuals with varying ages, gender, and health status, so it is perhaps not surprising the reported effects were conflicting. A better understanding of osteogenic effects of bFGF and BMP-2 is required to determine their potential in osteoporosis therapy. We proposed three aims for this study: to compare the dose-response relationships for the two growth factors to probe the relative potency of the proteins for osteogenesis; to determine the influence of a short (1-week) growth factor exposure duration to probe the lasting effects of the proteins' therapeutic action, and; to ascertain any changes in growth factor responsiveness with age.

4.2 Materials and Methods

Three series of experiments were conducted for the three purposes of this study. In the first series of experiments where the relative potency of growth factors were investigated, BMC from young rats (6 weeks) were treated with bFGF (2–50 ng/mL) and BMP-2 (50–500 ng/mL) for a duration of 3-weeks. In the second series of experiments

where the effect of growth factor exposure duration was investigated, BMC from young rats were treated with bFGF (2–50 ng/mL) and BMP-2 (50–500 ng/mL) combinations for a 1-week period, after which the cells were maintained in medium without the growth factors for a 3-week period. In the third series of experiments in which the effect of age on osteogenic response was investigated, the second series of experiments were repeated but by using BMC obtained from adult rats (32 weeks old). The DNA content, ALP activity and mineralization as a function of time were determined in all experiments.

We used female Sprague–Dawley rats from Biosciences (Edmonton, Alberta, Canada). Six-week-old and 32-week-old rats were shipped to the University of Alberta and the animals were housed for an additional 2 weeks for acclimatization to laboratory conditions. The rats were allowed free access to food and water and were kept on a 12:12 hours of light:dark cycle. All animal procedures were performed according to guidelines of the Canadian Council on Animal Care and were institutionally preapproved before the study onset.

We used Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS; without phenol red), penicillin (10,000 U/mL solution), streptomycin (10,000 μg/mL solution), and heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY). All tissue culture plastic ware was from Corning (Corning, NY). Recombinant bFGF was obtained from Peprotech Inc (Rocky Hill, NJ). Recombinant BMP-2 was obtained from an Escherichia coli expression system. Its activity was reported in the literature [10, 29]. A CyQUANT cell proliferation kit from Molecular Probes (Portland, OR) was used to quantitate DNA concentration in cell lysates. The tissue culture reagents dexamethasone, β-glycerol phosphate (β-GP), ascorbic acid, the

ALP substrate p-nitrophenol phosphate (p-NPP), and p-nitrophenol (p-NP) were from Sigma (St. Louis, MO) and they were used without further purification. The Sigma Kit #567 (Sigma) was used for calcium quantitation in solution.

The rats were sacrificed by CO_2 asphyxiation, and the BMC were isolated from both femurs and pooled to obtain a single cell suspension. The cells were isolated using aseptic techniques in a biological safety cabinet. The marrow was removed by cutting the femur at distal and proximal ends with a bone cutter and then was aspirated using a 5-mL syringe with an 18G needle. The bone marrow was flushed out with approximately 15 mL of DMEM containing 10% FBS, 50 mg/L ascorbic acid, 100 U/mL penicillin, and 100 μg/L of streptomycin (referred as basal medium). The cells were centrifuged (600 g for 10 minutes) and suspended in a small volume of fresh basal medium, and the cell density was determined with a hemocytometer. The centrifugation was performed to remove endogenous molecules, which was previously suggested to influence establishment of BMC colonies [31]. The bone marrow aspirate was seeded in 25 cm^2 flasks in duplicate.

The cells were allowed to attach and grow for a period of 7 days in 25-cm^2 flasks in duplicate. Afterwards the spent medium and unadherent cells were removed and the cell monolayer was washed with cold HBSS. The cells were trypsinized with 0.25% trypsin/EDTA, harvested and centrifuged (approximately 600 g), and subcultured on 25 cm^2 flasks for one additional week after 1:4 dilution of the obtained cell suspension. After this time period, the BMC was trypsinized, seeded in 24-well plates, and allowed to attach for 1 day in basal medium. The medium subsequently was changed to osteogenic media (basal–medium-containing FBS, ascorbic acid, penicillin, streptomycin, and

supplemented with 10 mM β-GP and 100 nM dexamethasone). Where indicated, the full osteogenic medium was diluted by 3/10 and 1/10 with the basal medium. The growth factors were added to the medium at desired concentrations (0–50 ng/mL for bFGF and 0–500 ng/mL for BMP-2). The choice of the growth factor concentrations was based on bFGF [33] and BMP-2 [27] studies that reported mitogenicity and differentiation effects, respectively, of rat BMC. The BMC were exposed to continuous bFGF and BMP-2 exposure for a 3-week period or a 1-week period followed by 3 weeks of culture in osteogenic medium without any growth factor supplements. In the former case, the medium was changed weekly with fresh addition of the growth factors. In the latter case, the medium was changed to the desired osteogenic media (without growth factors) on a weekly basis.

The cells in multiwell plates were harvested at predetermined time points to obtain quantitative measures of the chosen osteogenic parameters. In the case of continuous growth factor exposure, the cells were harvested after 1, 2, and 3 weeks of continuous growth factor treatment. In the case of short (1-week) growth factor exposure, the cells were harvested after 1, 2, and 3 weeks of the removal of the growth factors in media.

To determine ALP activity, BMC were washed with HBSS (×2) and lysed with 300 μL ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X; pH, 10.5). Two hours after lysis, 150 μL of lysed solution was added to 48-well plates, and 150 μL of 2 mg/mL ALP substrate (p-NPP) was added to the lysed cells to give a final concentration of 1 mg/mL pNPP. The changes in optical density ($\lambda_{absorbance}$: 405 nm) were determined in a multiwell plate reader at periodic intervals for up to 15 minutes. To

determine the DNA content in wells, the remaining cell lysis solution was frozen at -20°C, thawed at a suitable time, and analyzed with the CyQUANT DNA kit according to the manufacturer's instructions using a fluorescent plate reader ($\lambda_{excitation}$ at 480 nm, $\lambda_{emission}$ at 527 nm). A DNA standard provided with the CyQUANT kit was used to determine the DNA concentrations in cell preparations.

The wells containing the lysed BMC were washed with HBSS (x2) and treated with 0.25 mL of 0.5 M HCl for 4 to 6 hours to determine calcification. The aliquots of the solutions were used to quantify the amount of dissolved calcium using the Sigma diagnostic kit ($\lambda_{absorbance}$, 574 nm). Manufacturer supplied reference solutions were used for a calcium standard curve. In the first set of experiments BMC were first subjected to continuous bFGF and BMP-2 exposure for a duration of 3 weeks. The cells were treated with all combinations of bFGF (i.e., 0, 2, 10, and 50 ng/mL) and BMP-2 (i.e., 0, 50, 150, and 500 ng/mL) concentrations. In the second series of studies we use short-term (1 week) exposure of BMC to BMP-2 and bFGF and determined changes in the osteogenic parameters. The medium for these experiments was 3/10 osteogenic medium because it was sufficient to induce mineralization in continuous exposure experiments.

The relative ALP activity was expressed as the change in optical density of the wells per unit time (mAbs/minute), and further normalized with the DNA content of the wells. The level of calcification was summarized as the concentration of calcium (mg/dL) per well. All results were expressed as mean ± standard deviation (SD), and statistical differences ($p < 0.05$) among the study groups were analyzed by analysis of variance (ANOVA).

4.3 Results

4.3.1 Comparative Response of BMC to Growth Factors

BMC in full OM retained their viability for 2 weeks in the absence of bFGF. However, a reduction ($p < 0.05$) in cell viability was observed after 3 weeks based on the reduced DNA content of the BMC (Fig. 2-1A). The continuous bFGF exposure at 2–50 ng/mL resulted in better cell viability after 3 weeks, but did not affect the DNA content at previous assessment points (Fig. 2-1A). In 3/10 and 1/10 OM there was no differences in cell numbers for bFGF treated cells for all three assessment points. There was no loss of cell viability after 3 weeks, as observed with full OM (in Fig. 2-1A). The ALP activity in the absence of bFGF increased from 1 week to 3 weeks (Fig. 2-1B), but bFGF treatment (especially at 10 and 50 ng/mL) reduced the specific activity at each assessment point (Fig. 2-1B). Similar results were obtained with 3/10 and 1/10 OM. Abundant calcification was seen in 3/10 and full OM, and the level of mineralization progressively increased with culture time (Fig. 2-1C). With bFGF there was an accelerated mineralization at 10 ng/mL concentration, but an increase of bFGF concentration to 50 ng/mL led to reduced mineralization after 2 and 3 weeks. There was no calcification with 1/10 OM at any of the bFGF concentrations or time points.

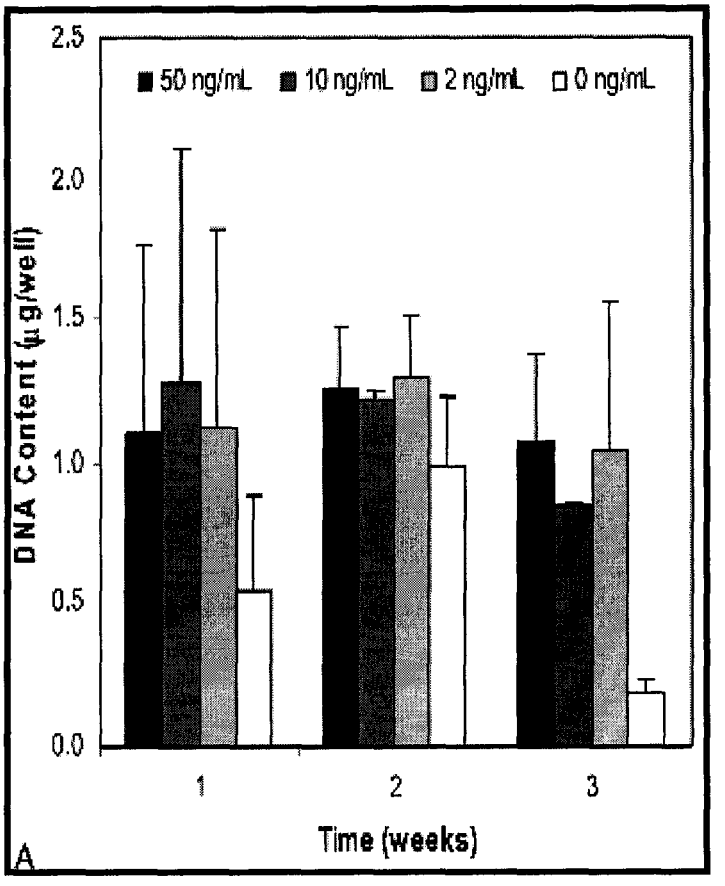

Figure 2-1A. Changes in the DNA content after 1, 2, and 3 weeks of continuous bFGF treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and allowed to attach for 1 day. Basic fibroblast growth factor was then added to wells at indicated concentrations. The amount of DNA (per well) was assessed after 1, 2, and 3 weeks. There were no apparent differences in DNA content for the bFGF treated BMC after 1 and 2 weeks, but a significant reduction of DNA content was observed after 3 weeks in the absence of bFGF treatment.

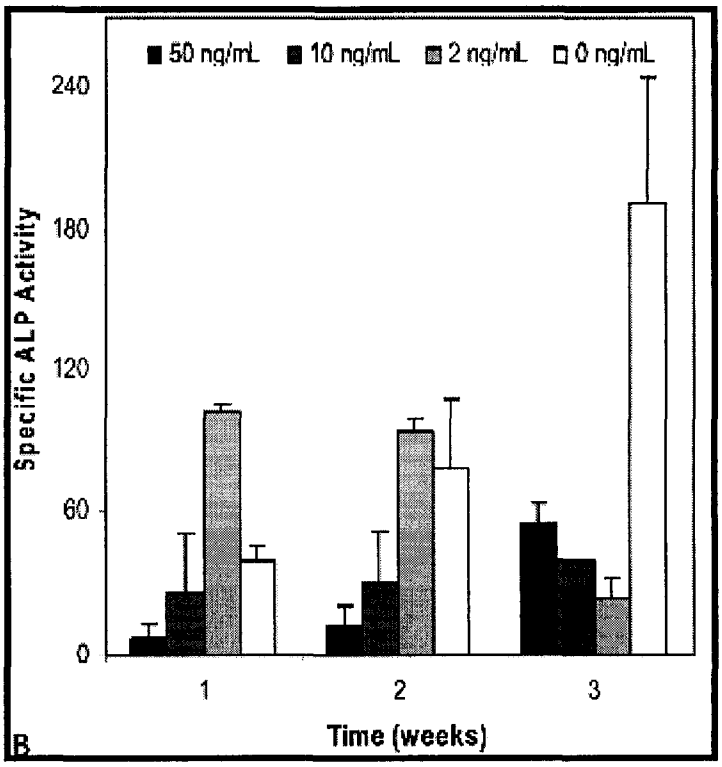

Figure 2-1B. Changes in ALP activity after 1, 2, and 3 weeks of continuous bFGF treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and allowed to attach for 1 day. Basic fibroblast growth factor was then added to wells at indicated concentrations. The amount of ALP activity (per well) was assessed after 1, 2, and 3 weeks. Basic fibroblast growth factor treatment of BMC, especially at the highest two concentrations (10 and 50 ng/mL, respectively), resulted in consistent reduction of ALP activity.

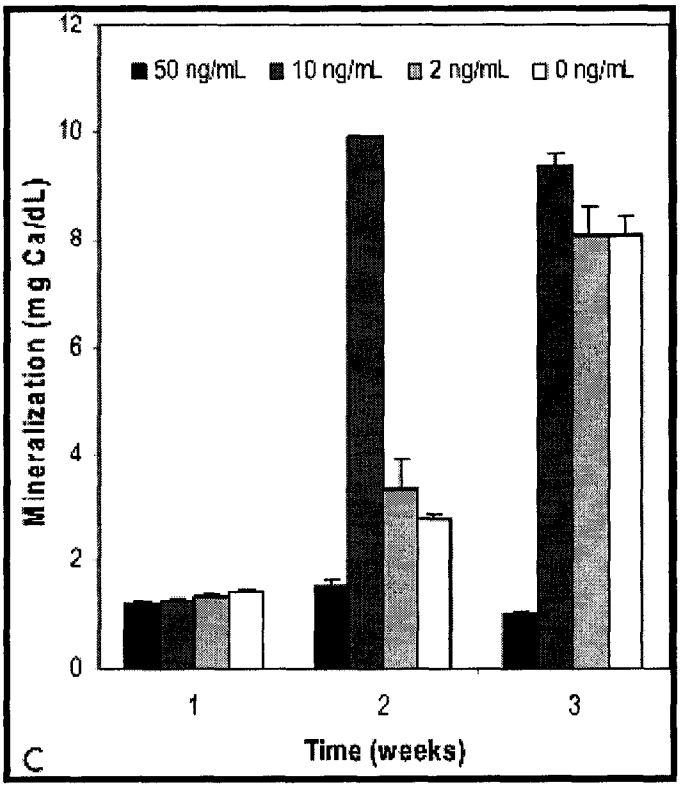

Figure 2-1C. Changes in mineralization after 1, 2, and 3 weeks of continuous bFGF treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and allowed to attach for 1 day. Basic fibroblast growth factor was then added to wells at indicated concentrations. The amount of mineralization (per well) was assessed after 1, 2, and 3 weeks. There was no mineralization in any of the BMC cultures after 1 week of culture, but significant mineralization was achieved after 2 weeks. Basic fibroblast growth factor at 10 ng/mL accelerated the extent of mineralization on Week 2, but the higher concentration of bFGF (50 ng/mL) resulted in a reduction of mineralization after 3 weeks.

Similar to the results with bFGF treatment, BMC in full OM retained their viability for 2 weeks, but a reduction in cell viability was observed after 3 weeks (Fig. 2-2A). The presence of BMP-2 did not influence the loss in viability in OM after 3 weeks (Fig. 2-2A). The BMC in 3/10 and 1/10 OM retained their viability throughout the 3-week study period, and the BMP-2 treatment of BMC did not have any influence on the DNA content. The ALP activity was increased as a function of BMP-2 concentration

when BMC was cultured in the full OM, but no clear effects were noted in other media (Fig. 2-2B). Similar to the results with the bFGF treatment, abundant calcification was seen in 3/10 OM and full OM, but not in 1/10 OM throughout the 3-week study period. The BMP-2 treatment at the highest concentrations (150 ng/mL and 500 ng/mL) stimulated mineralization in the full OM, but a clear dose response effect of BMP-2 treatment on mineralization was clearest in the 3/10 OM (Fig. 2-2C). This was the case for the 2-week and 3-week assessment points.

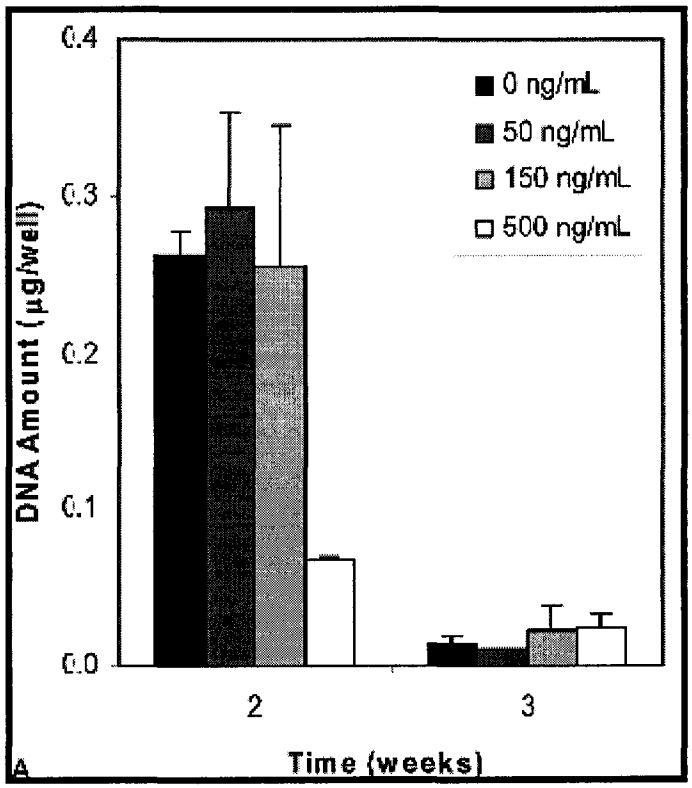

Figure 2-2A. Changes in the DNA content after 2 and 3 weeks of continuous BMP-2 treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and were allowed to attach for 1 day. Bone morphogenetic protein-2 was then added to wells at indicated concentrations. The amount of DNA (per well) was assessed after 2 and 3 weeks. A significant reduction of DNA content was observed after 3 weeks, irrespective of the BMP-2 concentration in the medium.

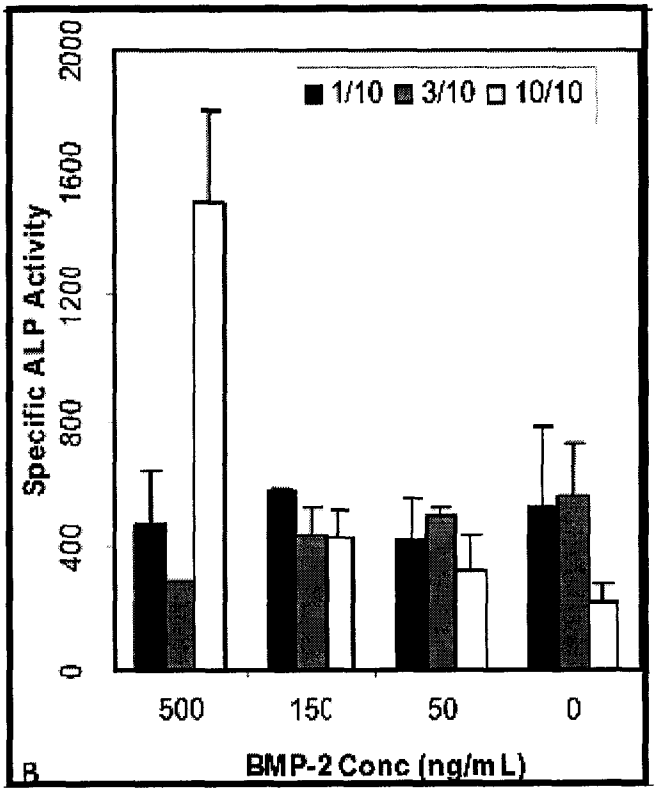

Figure 2-2B. Changes in the ALP activity after 2 and 3 weeks of continuous BMP-2 treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and were allowed to attach for 1 day. Bone morphogenetic protein-2 was then added to wells at indicated concentrations. The ALP activity (per well) was assessed after 2 and 3 weeks. The BMP-2 treatment of BMC at 500 ng/mL in full osteogenic medium gave an increased ALP activity, but no effect of BMP-2 was observed in 3/10 and 1/10 osteogenic medium.

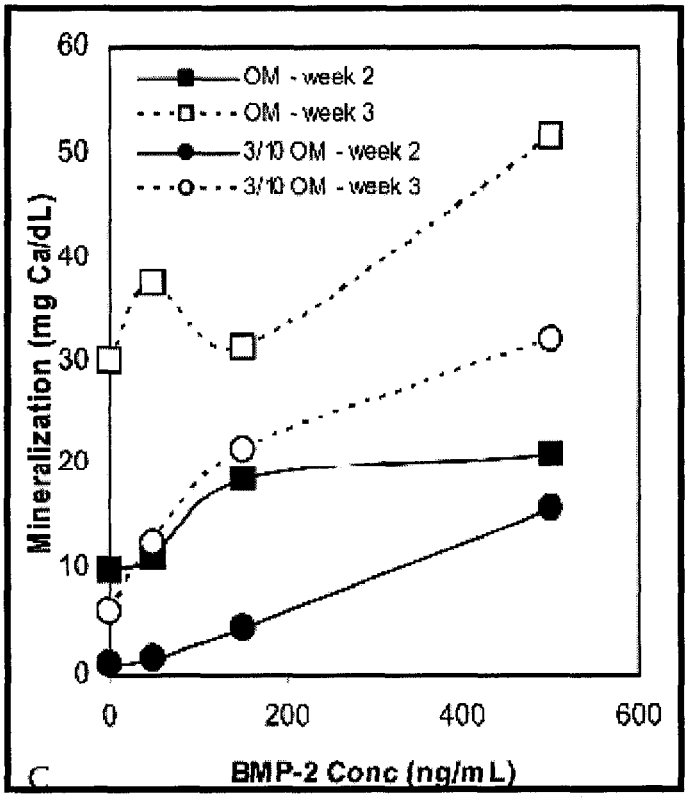

Figure 2-2C. Changes in mineralization after 2 and 3 weeks of continuous BMP-2 treatment in full osteogenic medium. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates and were allowed to attach for 1 day. Bone morphogenetic protein-2 was then added to wells at indicated concentrations. The amount of mineralization (per well) was assessed after 2 and 3 weeks. There was no mineralization in any of the BMC cultures after 1 week of culture (not shown), but significant mineralization was obtained after 2 weeks in full and 3/10 osteogenic media. The BMP-2 effect on mineralization was most obvious in 3/10 osteogenic medium, where the propensity of cultures to mineralize was low in the absence of BMP-2.

4.3.2 Short-Term Exposure to Growth Factors

BMP-2 generally led to a reduced cell numbers, which was most evident in the highest concentrations of BMP-2 (500 ng/mL) after 2 (Fig. 2-3B) and 3 weeks (Fig. 2-3C). For this BMP-2 concentration, bFGF increased the DNA content of BMC in the concentration range tested ($p < 0.01$ in Fig. 2-3A; $p < 0.05$ in Fig. 2-3B; $p < 0.01$ in Fig. 2-3C).

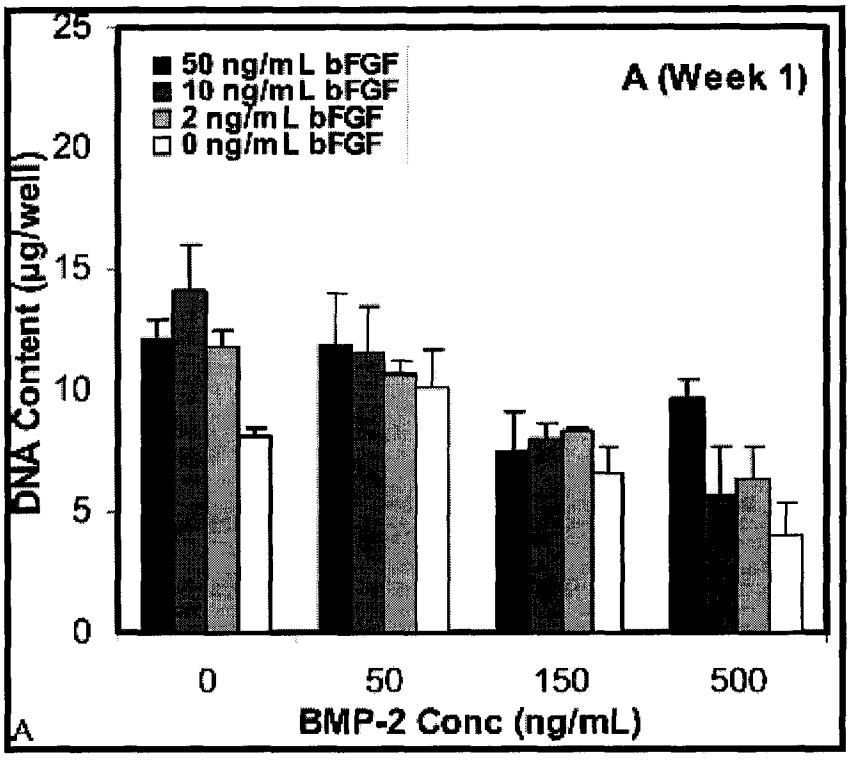

Figure 2-3A. Changes in the DNA content of BMC treated with bFGF and BMP-2 combinations at Week 1. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the DNA content per well was analyzed after 1, 2, and 3 weeks. There was a general tendency of decreasing DNA content per well as the concentration of BMP-2 was increased, and bFGF did not have a strong effect on the DNA content at each BMP-2 concentration.

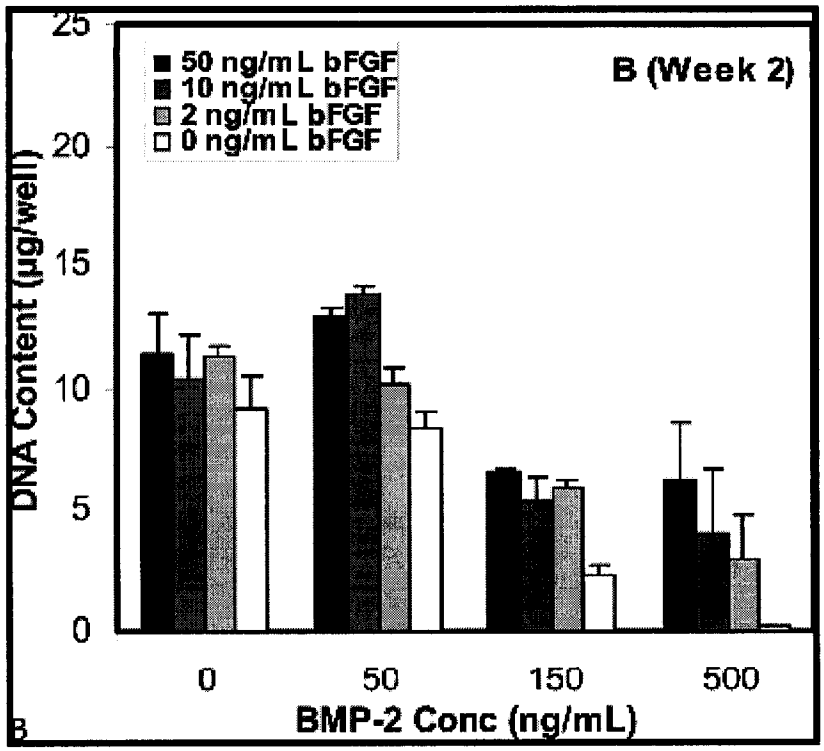

Figure 2-3B. Changes in the DNA content of BMC treated with bFGF and BMP-2 combinations at Week 2. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the DNA content per well was analyzed after 1, 2, and 3 weeks. A detrimental effect of BMP-2 on DNA content was evident especially at the highest concentration (500 ng/mL) of BMP-2. Co-treatment with bFGF resulted in a concentration dependent increase in the DNA content, especially visible at the higher BMP-2 concentration.

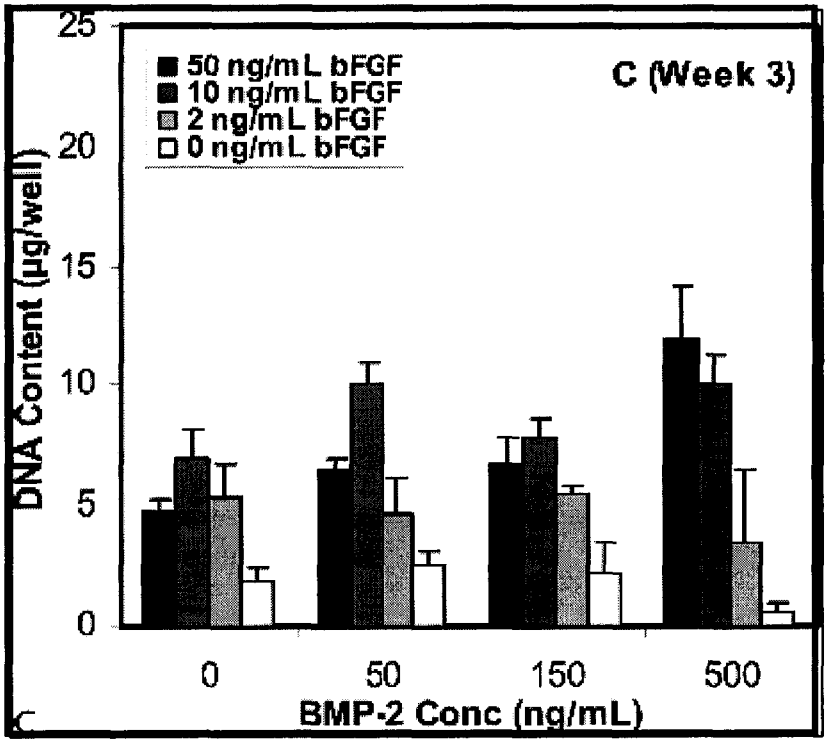

Figure 2-3C. Changes in the DNA content of BMC treated with bFGF and BMP-2 combinations at Week 3. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the DNA content per well was analyzed after 1, 2, and 3 weeks. As seen at Week 2, BMP-2 at the highest concentration resulted in reduced DNA content per well, which was increased by co-treatment with bFGF.

There was no clear effect of bFGF and BMP-2 on ALP activity after 1 and 2 weeks (Fig. 2-4 A-C). BMP-2 did not lead to a reproducible increase in the ALP activity, and bFGF did not appear to consistently increase or decrease the measured ALP activity. Only on Week 3 did bFGF reduce the ALP activity in the presence of 50, 150, and 500 ng/mL BMP-2.

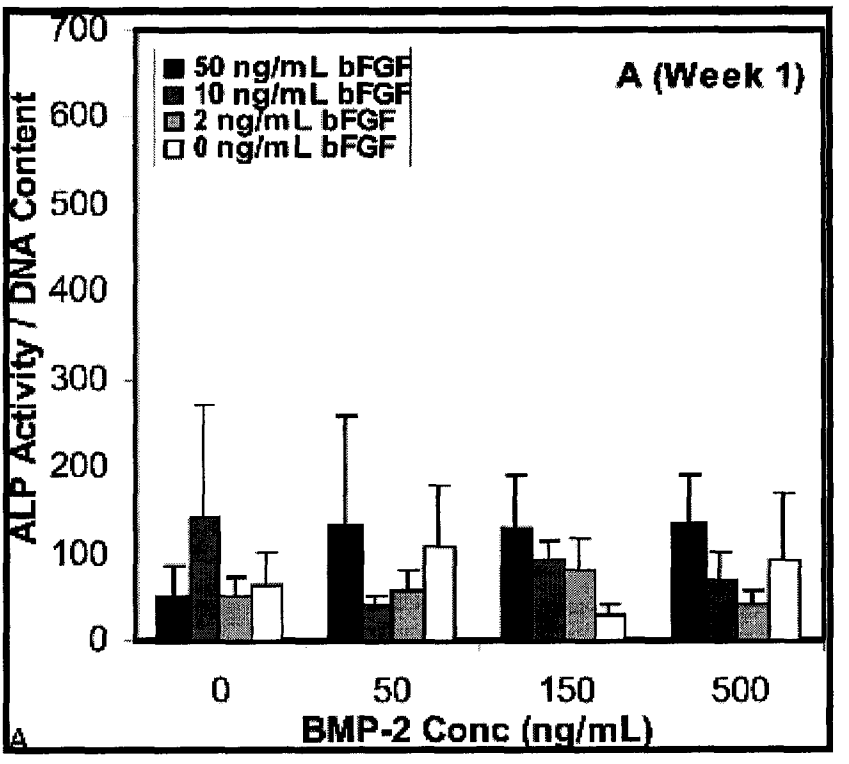

Figure 2-4A. Changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations at Week 1. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. There was no clear effect of bFGF, BMP-2, or bFGF and BMP-2 co-treatment on the ALP activity of BMC at Week 1.

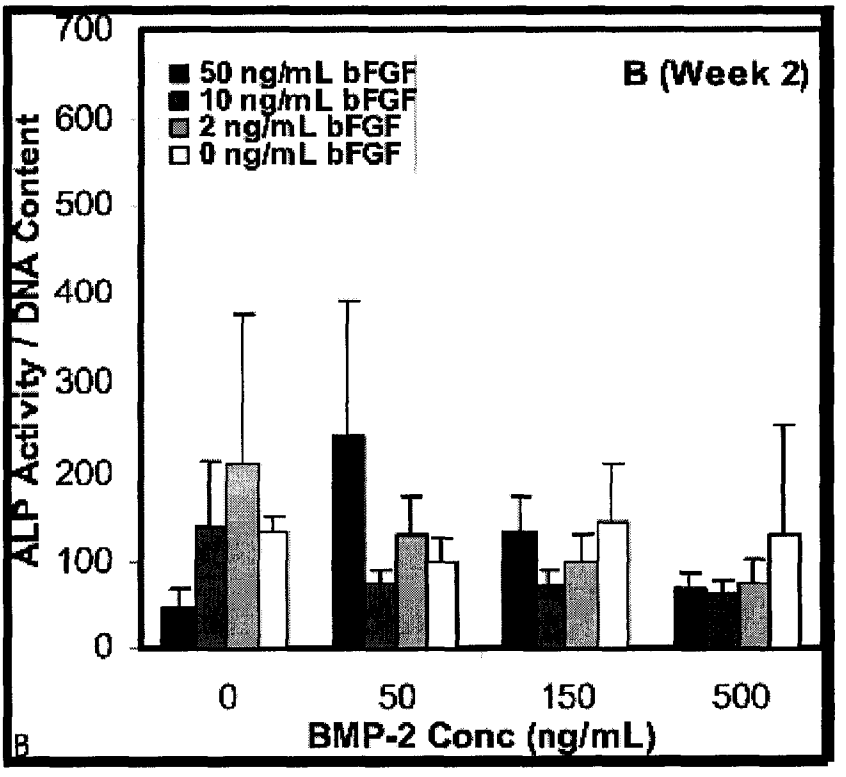

Figure 2-4B. Changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations at Week 2. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. There was no clear effect of bFGF, BMP-2, or bFGF and BMP-2 co-treatment on the ALP activity of BMC at Week 2.

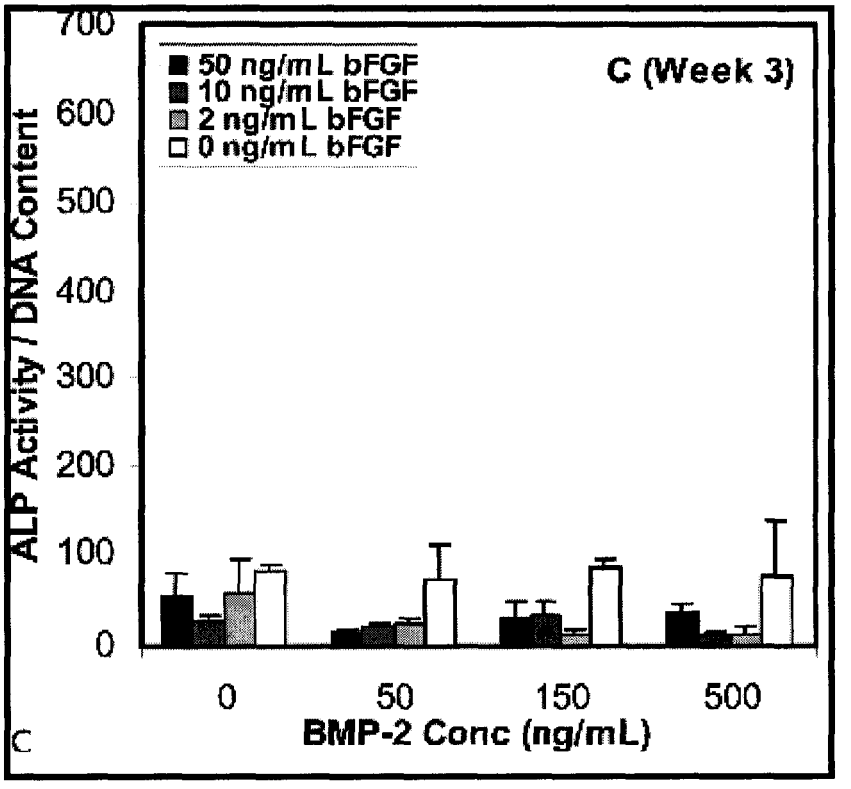

Figure 2-4C. Changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations at Week 3. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. BMP-2 in the absence of bFGF did not influence the ALP activity, but bFGF (2-50 ng/mL) in the presence of 50, 150, and 500 ng/mL BMP-2 reduced the ALP activity after 3 weeks.

The effects of bFGF and BMP-2 treatment on BMC mineralization are summarized in Fig. 2-5. As before, there was a progressive increase in the level of mineralization as a function of time. The extent of mineralization was increased with an increase in BMP-2 concentration, particularly after 1 (Fig. 2-5A; $p < 0.05$) and 2 (Fig. 2-5B; $p < 0.05$) week of stopping the growth factor treatment. After 3 weeks, BMC underwent mineralization even without the BMP-2 treatment. No effect of bFGF was

seen on the level of mineralization, as the extent of mineralization was generally the same for a given BMP-2 concentration.

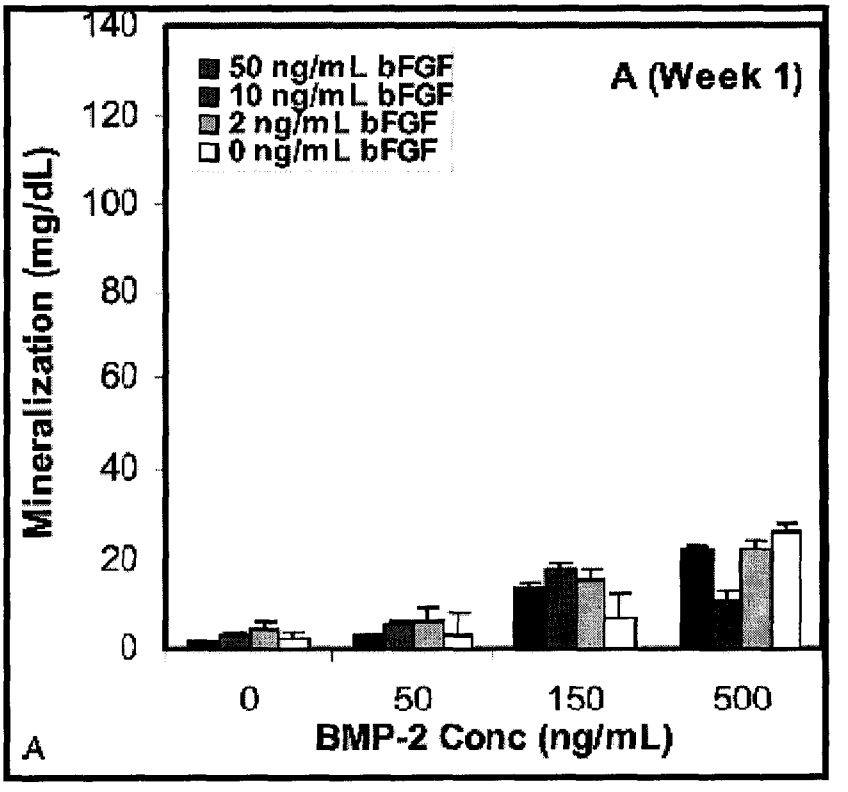

Figure 2-5A. Changes in the mineralization of BMC treated with bFGF and BMP-2 combinations at Week 1. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. There was a general tendency of increasing mineralization of BMC with the increasing BMP-2 concentration at Week 1. There was no effect of bFGF on the extent of mineralization.

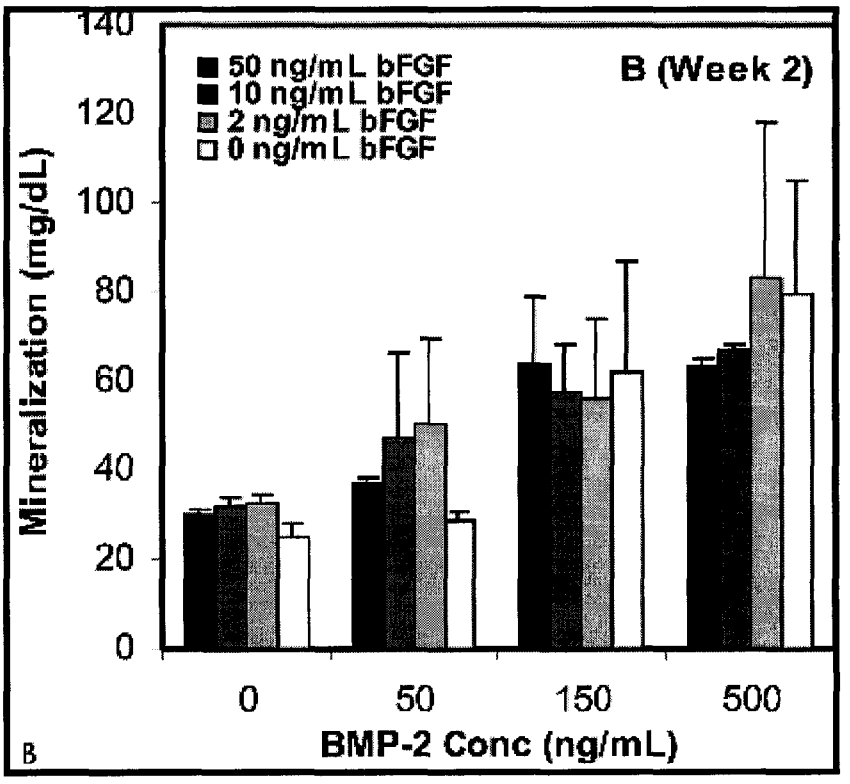

Figure 2-5B. Changes in the mineralization of BMC treated with bFGF and BMP-2 combinations at Week 2. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. There was a general tendency of increasing mineralization of BMC with the increasing BMP-2 concentration at Week 2. There was no effect of bFGF on the extent of mineralization.

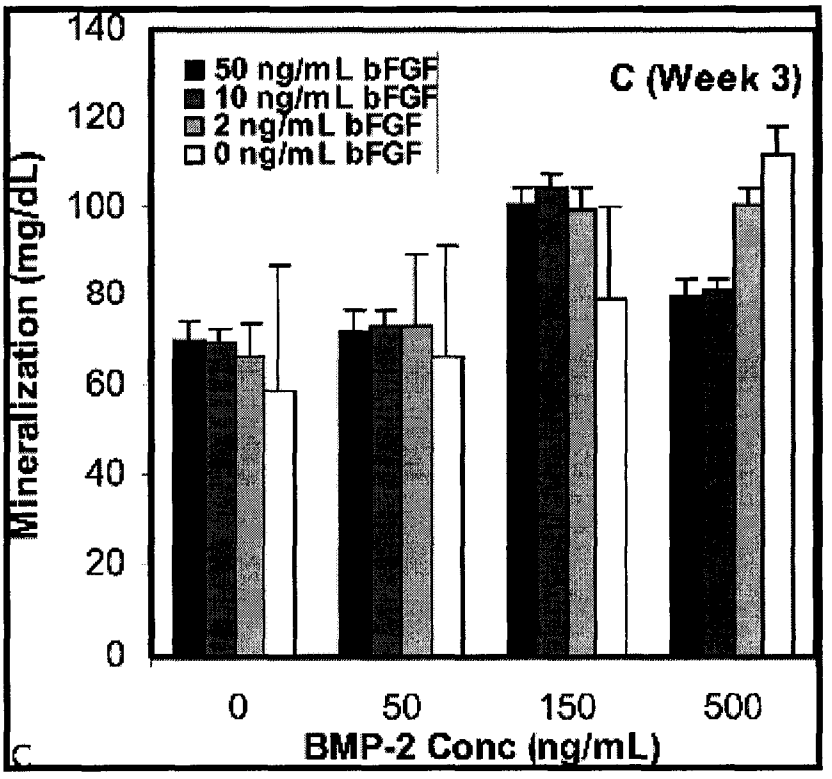

Figure 2-5C. Changes in the mineralization of BMC treated with bFGF and BMP-2 combinations at Week 3. The BMC (obtained from 8-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. The effect of BMP-2 on mineralization was reduced because of the higher level of mineralization in the absence (0 ng/mL) of BMP-2. The increased mineralization at 500 ng/mL BMP-2 concentration was attenuated in the presence of 10–50 ng/mL bFGF.

4.3.3 Growth Factor Effects on BMC from Older Rats

BMP-2 treatment (in the concentration range tested) did not affect the cellular DNA amount (Fig. 2-6). Similarly, bFGF did not affect the DNA content of the BMC. The DNA content of the BMC derived from the older rats was generally lower than the DNA content of BMC from younger rats (compare Figs 2-3A vs. 2-6A, 2-3B vs. 2-6B and 2-3C vs. 2-6C).

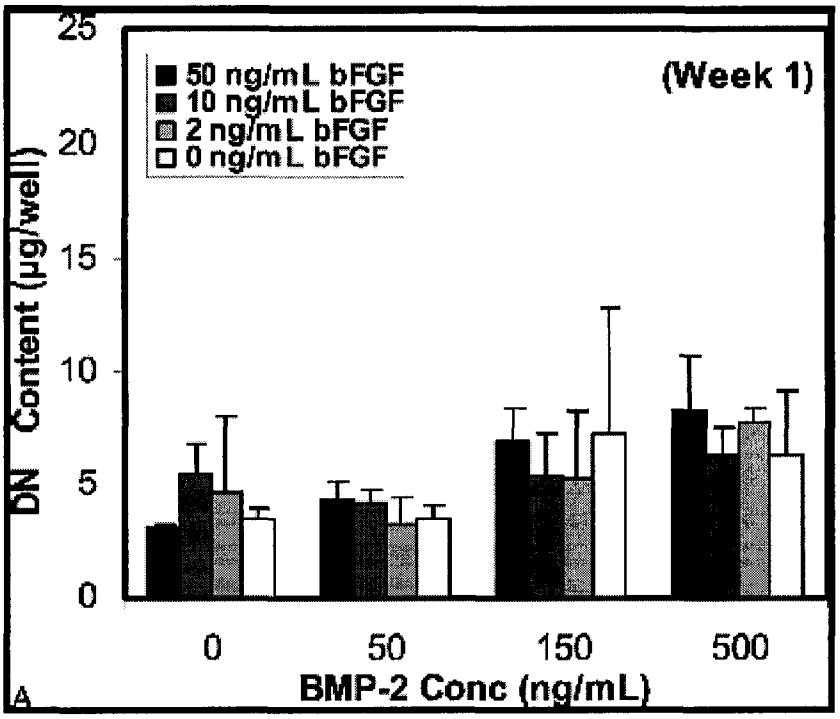

Figure 2-6A. Changes in the DNA content of BMC treated with bFGF and BMP-2 combinations at Week 1. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the DNA content per well was analyzed after 1week. There was no clear effect of bFGF or BMP-2 on the DNA content per well. Note that the viability of BMC was retained in these cultures, unlike the BMC obtained from younger rats.

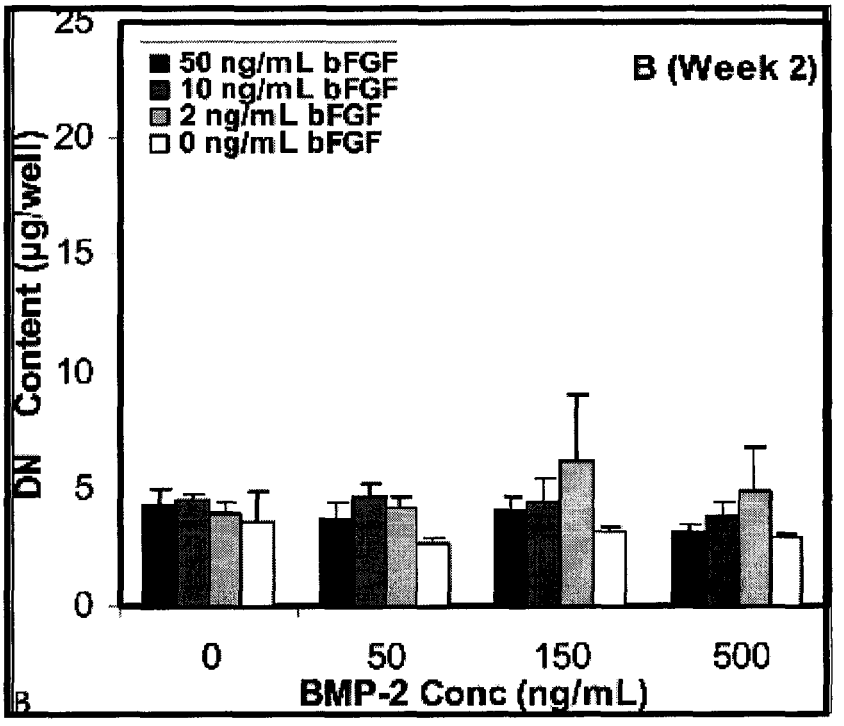

Figure 2-6B. Changes in the DNA content of BMC treated with bFGF and BMP-2 combinations at Week 2. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the DNA content per well was analyzed after 2 weeks. There was no clear effect of bFGF or BMP-2 on the DNA content per well. Note that the viability of BMC was retained in these cultures, unlike the BMC obtained from younger rats.

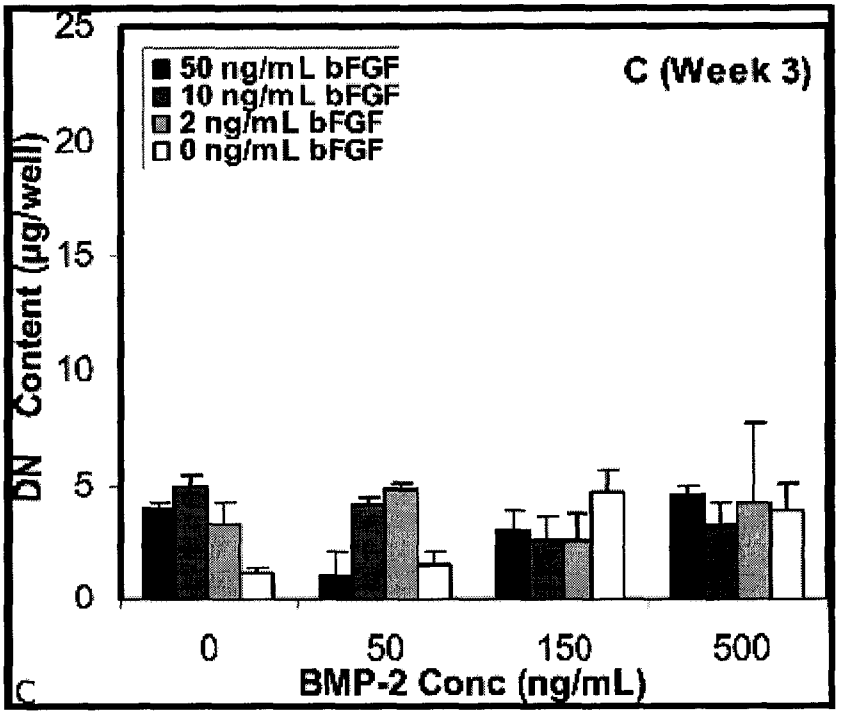

Figure 2-6C. Changes in the DNA content of BMC treated with bFGF and BMP-2 combinations at Week 3. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the DNA content per well was analyzed after 3 weeks. There was no clear effect of bFGF or BMP-2 on the DNA content per well. Note that the viability of BMC was retained in these cultures, unlike the BMC obtained from younger rats.

The ALP activity of BMC from older rats was not dependent on BMP-2 or bFGF treatment on Week 1 (Fig. 2-7A). The bFGF treatment of BMC was stimulatory on Week 2 (Fig. 2-7B), especially for lower concentrations of BMP-2 (0 ng/mL and 50 ng/mL). Similarly, bFGF was stimulatory on ALP activity for the lower concentrations (0, 50, and 150 ng/mL) of BMP-2 on Week 3 (Fig. 2-7C).

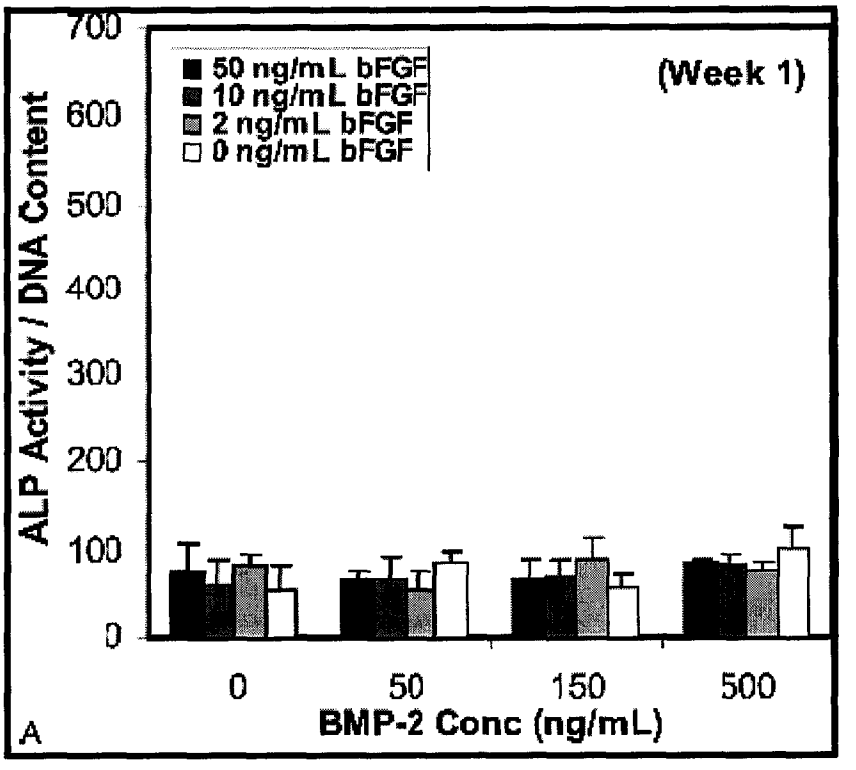

Figure 2-7A. Changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations at Week 1. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. There was no clear effect of bFGF, BMP-2 or bFGF and BMP-2 co-treatment on the ALP activity of BMC at Week 1. Bone morphogenetic protein-2 in the absence of bFGF did not affect ALP activity.

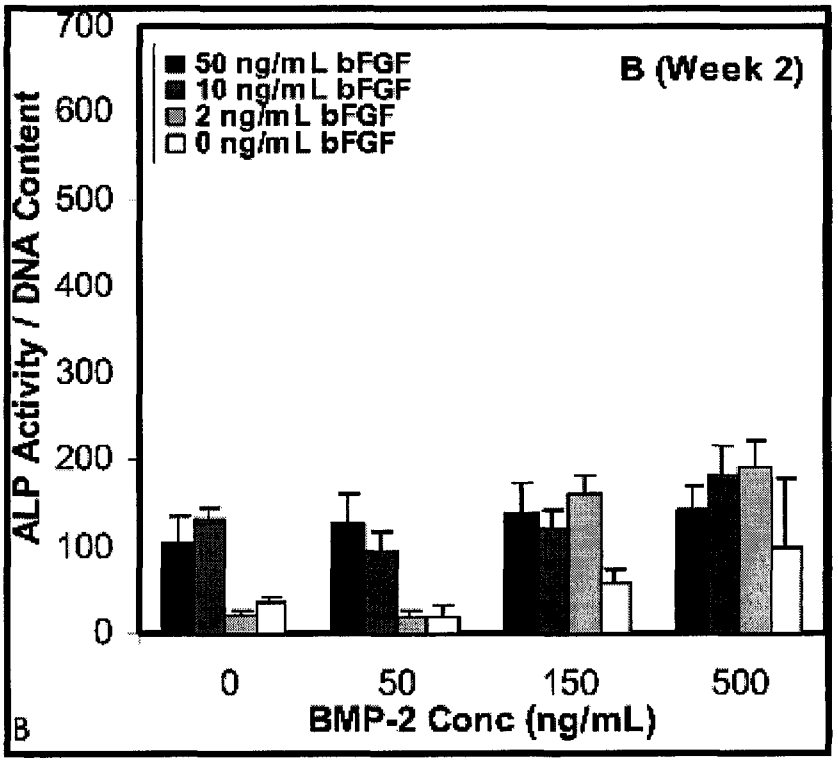

Figure 2-7B. Changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations at Week 2. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. There was no clear effect of bFGF, BMP-2 or bFGF and BMP-2 co-treatment on the ALP activity of BMC at Week 2. The only stimulatory effect of bFGF was seen at low BMP-2 concentrations (0 and 50 ng/mL). Bone morphogenetic protein-2 in the absence of bFGF did not affect ALP activity.

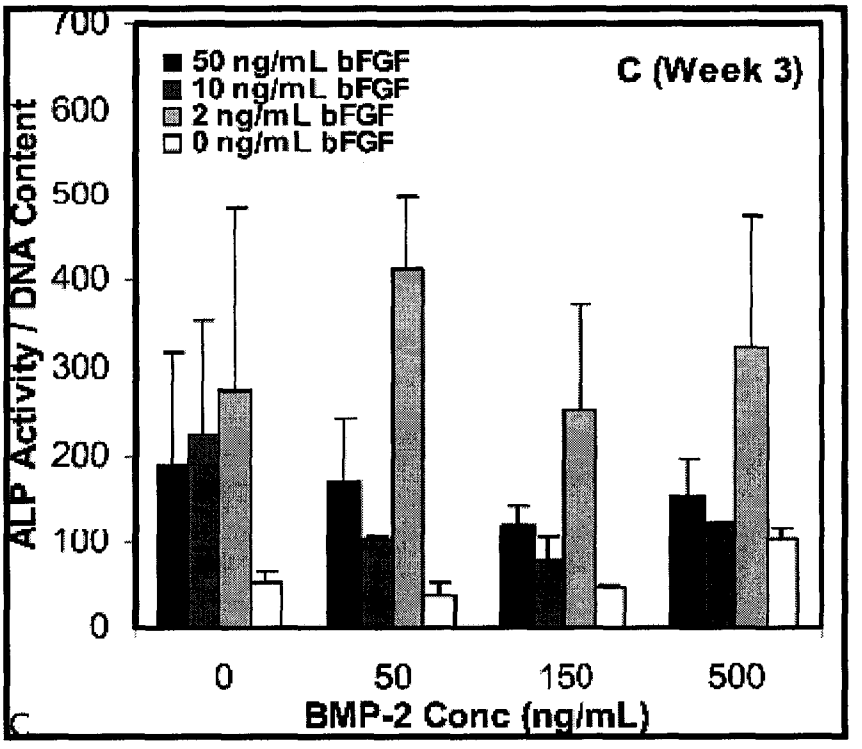

Figure 2-7C. Changes in the ALP activity of BMC treated with bFGF and BMP-2 combinations at Week 3. The BMC (obtained from 32-week-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors, and the ALP activity was analyzed after 1, 2, and 3 weeks and normalized with the DNA content per well. At Week 3, bFGF at 2 ng/mL appeared to stimulate the ALP activity at all concentration of BMP-2, but higher concentration of bFGF did not affect the ALP activity. Bone morphogenetic protein-2 in the absence of bFGF did not affect ALP activity.

We found a clear effect of BMP-2 on the extent of mineralization (Fig. 2-8). As BMP-2 concentration increased there was a general increase ($p < 0.01$ at week 1, week 2 and week3) in the extent of mineralization at all assessment times. At the highest concentration of BMP-2 there was no clear effect of bFGF on the extent of mineralization after 1, 2, and 3 weeks. With 150 ng/mL, cotreatment with bFGF increased the extent of mineralization in a dose dependent manner. Mineralization at 0 mg/mL and 50 ng/mL

BMP-2 was minimal after 1 and 2 weeks (Fig. 2-8A and 2-8B), and the extent of mineralization on week 3 were not different between these two groups (Fig. 2-8C).

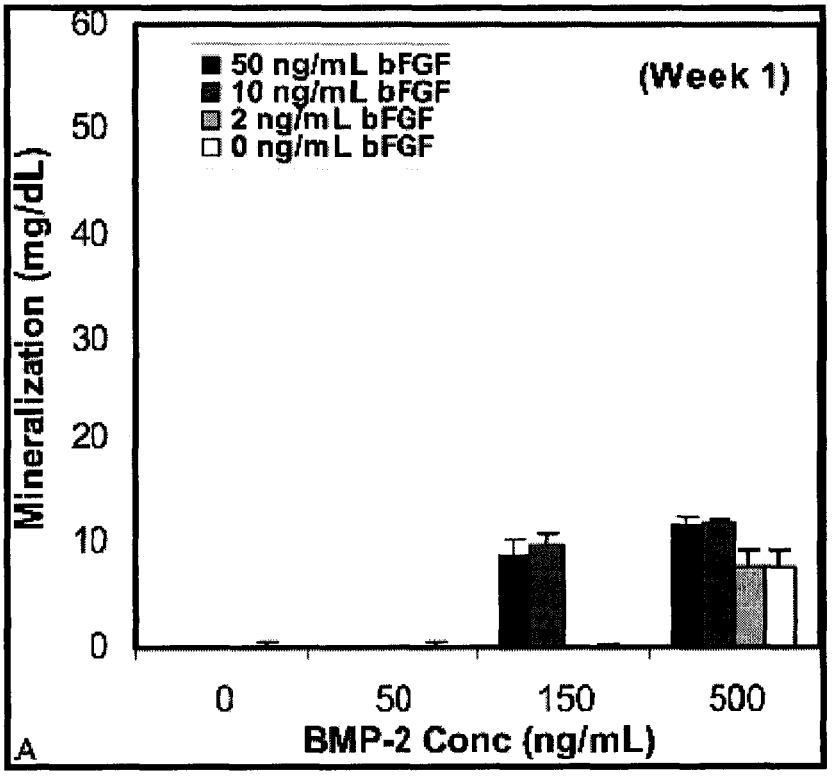

Figure 2-8A. Changes in the mineralization of BMC treated with bFGF and BMP-2 combinations at Week 1. The BMC (obtained from 32-weeks-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. Clear mineralization of BMC was evident only at the highest concentrations (150 and 500 ng/mL) of BMP-2 on Week 1. Where BMP-2 induced mineralization was retarded (at 150 ng/mL), bFGF at 10 ng/mL and 50 ng/mL had a stimulatory role in inducing mineralization. Basic fibroblast growth factor alone was not sufficient for inducing mineralization in the absence (0–50 ng/mL) of BMP-2.

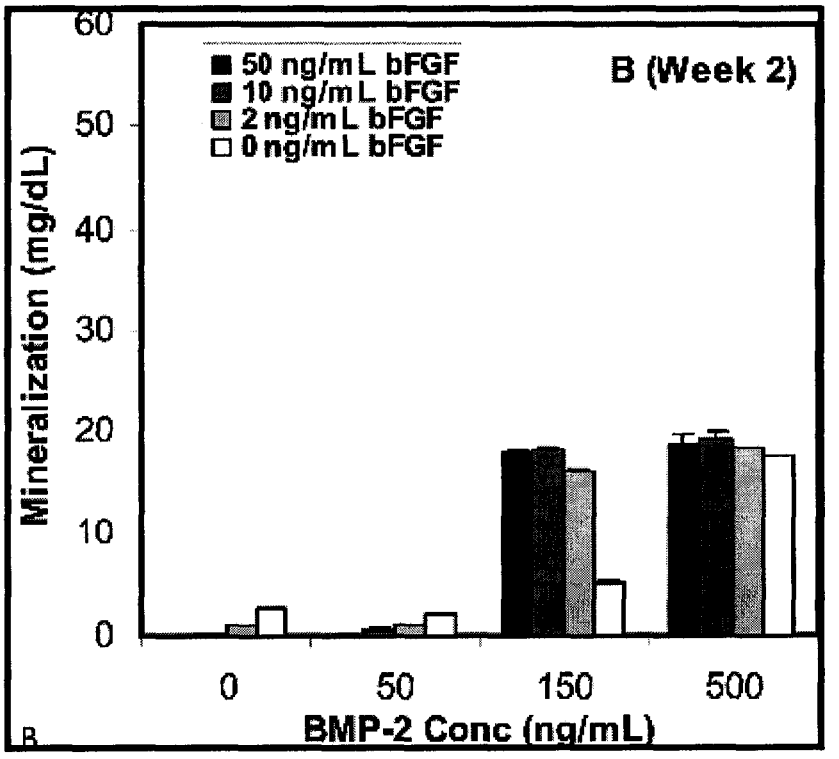

Figure 2-8B. Changes in the mineralization of BMC treated with bFGF and BMP-2 combinations at Week 2. The BMC (obtained from 32-weeks-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. Clear mineralization of BMC was evident only at the highest concentrations (150 and 500 ng/mL) of BMP-2 on Week 2. Where BMP-2 induced mineralization was retarded (at 150 ng/mL), bFGF at 10 ng/mL and 50 ng/mL had a stimulatory role in inducing mineralization. Basic fibroblast growth factor alone was not sufficient for inducing mineralization in the absence (0–50 ng/mL) of BMP-2.

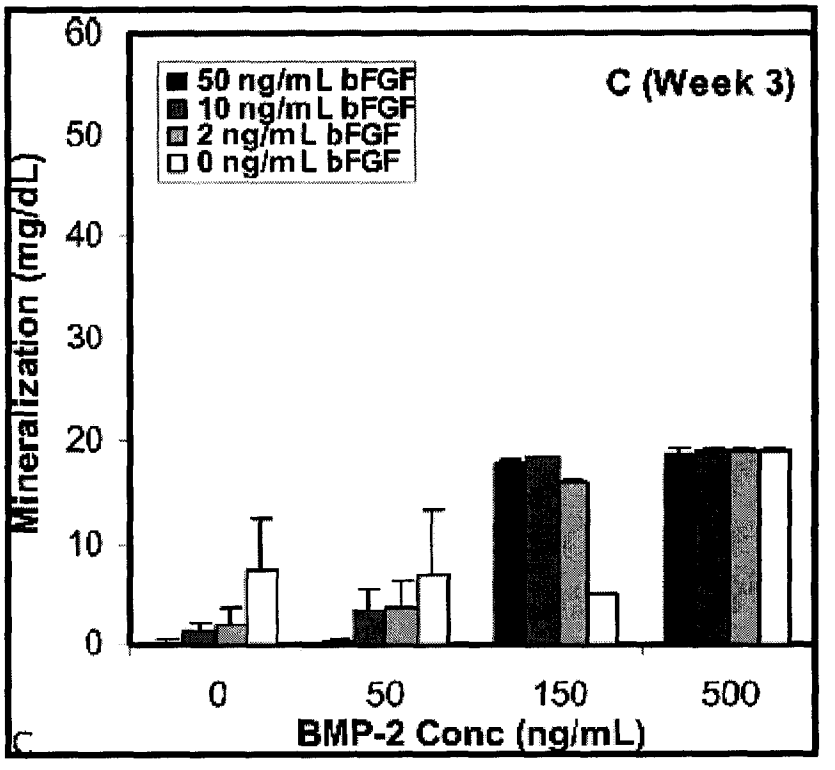

Figure 2-8C. Changes in the mineralization of BMC treated with bFGF and BMP-2 combinations at Week 3. The BMC (obtained from 32-weeks-old rats) were seeded in 24-well plates in 3/10 osteogenic medium and allowed to attach for 1 day. The growth factor combinations were then added to the wells for 1 week. The medium was changed to remove the growth factors and the calcium content per well was analyzed after 1, 2, and 3 weeks. As with Weeks 1 and 2, at Week 3 strong mineralization was evident only at the highest BMP-2 concentrations; bFGF had a stimulatory role on mineralization when BMP-2 concentration was submaximal; and bFGF had no effect of mineralization when BMC were exposed to the highest BMP-2 concentration (500 ng/mL).

4.4 Discussion

We investigated the responsiveness of BMC to two prototypical growth factors, bFGF and BMP-2. The culture conditions were optimized to allow cellular attachment and growth during the initial phase of BMC attachment and proliferation. Dexamethasone and β-GP had to be eliminated from the initial plating medium because of their inhibitory effects on cell growth [6]. Although the synthetic glucocorticoid dexamethasone and

β-GP combination is routinely used for osteogenic differentiation, detrimental effects of dexamethasone and inorganic phosphate have been reported [18]. Inorganic phosphate (in the form of $H_2PO_4^-$, the likely hydrolysis product of a β-GP) was found to induce apoptosis [18], and dexamethasone was shown to decrease the initial colony establishment in rat [1, 26] and human BMC dose dependently [16]. We found such a detrimental effect of the osteogenic supplements previously and identified β-GP as the primary source [6]. Full osteogenic medium (10 mM β-GP) gave reduced levels of cell numbers (i.e., DNA content), and diluting the concentrations of the osteogenic supplements in the media alleviated the loss in cellular viability (i.e., loss in DNA content). An osteogenic medium with 3 mM/30 nM β-GP/dexamethasone combination did not have any apparent detrimental effects on cell viability for a 4-week culture period (1 week of growth factor exposure + 3 weeks of subsequent culture without growth factors), except when the cells were exposed to the highest concentration (500 ng/mL) of BMP-2. The differences in viability observed in this study with 3 versus 10 mM β-GP were consistent with the differences in viability obtained by using human bone-derived osteoblast like cells [18]. Given the stimulatory effect of high concentration of BMP-2 on mineralization, it is likely that the cell viability might have been reduced as a result of extracellular mineralization. The bFGF was found to be beneficial in increasing viable cell numbers (based on DNA amounts recovered per well), presumably because of the mitogenic effect of bFGF on a subpopulation of BMC. It must be stressed that the beneficial effect of bFGF on cell numbers was observed under some conditions, and was not universal (e.g., no benefit of bFGF was seen in Fig 2-1A).

As one would expect, results from *in vitro* cell cultures should be interpreted with caution. This study reported cellular responses in the absence of two critical variables present in the endogenous bone marrow environment. One variable not incorporated into our cultures is the soluble factors (e.g., hormones and other peptide-based regulators) that may modulate the cellular responses to bFGF and BMP-2 exposure. The other variable is the three-dimensional nature of the cellular environment *in vivo*. It was recently recognized that BMC grown within a 3-dimensinal configuration displays a differential response to growth factors, such as BMP-2 [7] and Transforming Growth Factor-β [9], as compared to the cells grown on two-dimensional surfaces. The results reported in our culture system should be considered as a first line approximation to the possible *in vivo* effects of the growth factor response.

Basic fibroblast growth factor and BMP-2 were capable of stimulating mineralization under certain culture conditions, but differences in their effects were evident. Irrespective of the duration of exposure, a clear dose-response was consistently obtained for BMP-2, which gave a progressively higher amount of mineralization as its concentration was increased. The BMP-2 appeared to act as osteogenic signal throughout the concentration range tested. It increased the mineral deposition without affecting cell numbers. This was consistent with the literature on the bioactivity of this morphogenetic protein [5, 27, 23]. The bFGF exhibited an optimal concentration of approximately 10 ng/mL for stimulation of mineralization for continuous exposure, after which a detrimental effect on mineralization was observed. It is likely that bFGF might directly inhibit the osteogenic activity of differentiated osteoblasts at higher concentrations. bFGF may also increase the population of other lineage cells at higher concentrations because

multiple cell lineages share the same pluripotent precursor cells as the osteoblasts in the bone marrow environment [25]. Further studies will be needed to differentiate between the two possibilities. BMP-2 was stimulatory on BMC after a short time (1-week) and continuous exposure, but the effects of bFGF were evident only after continuous treatment of BMC. There were no effects after the short time exposure. This indicated a lack of memory effects in the culture system utilized in this study. The stimulatory effect of bFGF on osteogenic colony formation (based on increased calcium positive, collagen positive, and ALP positive colonies form the BMC aspirates) has been reported to take least 5 days of continuous BMC exposure [31]. A clear distinction was the concentration difference required between the two growth factors. The well established morphogen BMP-2 exhibited its full osteogenic effect at 500 ng/mL, but bFGF required a much lower concentration (approximately 10 ng/mL) to exhibit its osteogenic effect. This aspect of bFGF action is beneficial when one considers the need to administer substantial amounts of the growth factor for systemic stimulation of bone formation. The lower the dose required for an effect on BMC, and the lower the side effects likely are to be observed at extraskeletal sites [17].

Differences in their mechanisms of action make bFGF and BMP-2 likely to act on different cellular pathways involved on osteogenesis, and they may act synergistically when co-delivered at skeletal sites. The bFGF and BMP-2 combination has been shown to act synergistically in several systems, including osteogenic differentiation of BMC in culture [5], bone deposition by the growth factor treated and transplanted BMC in vivo [5], and *de novo* osteoinduction after intramuscular [4] and subcutaneous [32] delivery of the growth factor combination. The co-treatment regimen used in our study was intended

to reveal if there was such a synergistic effect on BMC. No such effect from bFGF was observed on the BMP-2 responses (i.e., ALP activity and mineralization) of BMC from young rats. A synergistic effect was seen on the BMC of older rats. This was evident on the BMP-2 induced ALP activity and mineralization for lower BMP-2 concentrations (50 ng/mL and 150 ng/mL, respectively; Fig 2-8), and not for the higher concentration of 500 ng/mL. It was indicative of the synergistic effect manifesting itself only within a narrow range of growth factor concentrations, and may not necessary extent to all concentrations of the growth factors. This also occurred when bFGF and BMP-2 were codelivered subcutaneously in a rat ectopic model. Low doses of bFGF were stimulatory on BMP-2 induced ALP activity and *de novo* bone deposition, but high concentrations of bFGF were inhibitory [4]. It is likely that such biphasic interactions might also occur when BMC are exposed to the systemically administered growth factors. Our experiments may help identify the appropriate concentrations of growth factors for an optimal stimulation of bone deposition.

We also compared the response to growth factors when BMC were derived from young or older rats. The osteogenic potential of bone marrow environment generally declines with age [8, 20, 2]. This was similar to the DNA assessment in our study, which indicated a lower cell numbers emanating from the BMC of older rats. Although we observed a similar level of specific ALP activity in young and old rats, the mineralization with BMC from old rats was less than the mineralization with BMC from younger rats. The BMC of old rats did not calcify in the absence of BMP-2 (compare Figs 2-5, 2-8), and the best calcification with 500 ng/mL BMP-2 concentration was approximately five fold less with the BMC from older rats after 3 weeks of culture. bFGF did not appear to

affect the mineralization of BMC from young rats, but was stimulatory in increasing the mineralization for BMC of older rats, especially at lower concentrations of BMP-2. Our results were similar to Kotev-Emeth et al, [11] who used an animal model similar to ours (Sprague-Dawley rats of 1.5 months versus 9 months). They observed a decrease in *in vitro* cell growth for the 9-month-old rats, but BMC from these rats were more responsive to bFGF compared with the BMC from younger rats, which did not exhibit a bFGF response [11]. The bFGF stimulation of bone-like nodules (Alizarin-Red stained area) were also more substantial in the aged rats [11]. These observations suggest that osteogenic factors might be more beneficial for BMC at an advanced age. It will be interesting to see if this is true for BMC even for older rats, as an equivalent age for onset of osteoporosis is older than the 8-month-old to 9-month-old rats [11].

Our findings indicated that osteogenic supplements β-GP/dexamethasone at 10 mM/100 nM were detrimental on BMC viability after 3 weeks of culture. bFGF was beneficial in increasing cell viability under mineralizing conditions, presumably by stimulating cell proliferation. Whereas continuous or short-term exposure to BMP-2 resulted in enhanced mineralization in either conditions, bFGF exerted its stimulatory effect in a narrow range of concentrations (approximately 10 ng/mL) and only after continuous exposure to BMC. The clinical utility of the latter growth factor, therefore, is likely to require continuous administration, whereas BMP-2 administration could be shorter duration. Co-treatment of BMC with BMP-2 and bFGF did not indicate a synergistic role of the bFGF on BMP-2 induced ALP activity and mineralization with BMC from young rats, but it was evident with BMC from older rats for certain lower concentrations of BMP-2. The BMC from older rats is more representative of

osteoporosis patients, and this result indicates the possibility of utilizing the synergistic effects of bFGF and BMP-2 in a clinical scenario. Our results indicated differences in the osteogenic response depending on the age of rats from which the BMC were obtained, and concentration and the exposure duration of the growth factors. It will be important if such effects could be observed *in vivo* (i.e., if the cells in the native bone marrow environment display such differential effects after growth factor treatments). Our future studies are designed with this in mind.

4.5 References

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CHAPTER III

OSTEOGENIC RESPONSE OF BONE MARROW STROMAL CELLS FROM NORMAL AND OVARIECTOMIZED RATS TO LOW DOSE bFGF TREATMENT[3]

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5.1 Introduction

The bone marrow comprises of two main cell types, the stromal cells and the hematopoietic cells, each with distinct lineages. The stromal cells give rise to a variety of mesenchymal cells including osteoblasts, chondrocytes, myoblasts, adipocytes and reticular cells [1]. Bone marrow stromal cells (BMSC) are a well-recognized source of osteoprogenitor cells, which contribute to the maintenance of the osteoblast population at the skeletal tissues. The bone mass is maintained by the intrinsically-coupled resorption process arising from the osteoclastic activity, and the formation process arising from the osteoblastic activity. Estrogens depletion, which is typical of post-menopause, is found to accelerate the bone remodelling process, causing higher levels of bone resorption as compared to bone formation, leading to a 'net' loss in bone mass. The slow loss of bone tissue over a long period ultimately leads to significantly lower bone mineral density and increased fragility, making the bone susceptible to spontaneous fractures [2]. Interventions based on stimulation of BMSC towards osteoblastic phenotype, so called anabolic therapies, are being pursued for treatment of clinical conditions characterized by reduced bone mineral density.

Several *in vitro* as well as *in vivo* studies have identified basic fibroblast growth factor (bFGF) to be a potent anabolic agent on bone tissue. bFGF has been observed to significantly increase the proliferation, differentiation, and subsequent bone matrix synthesis in rat BMSC culture [3-5]. Short-term treatment of mouse BMSC with bFGF for the first 3 days in culture, led to an increase in the number, size, and mineralization of alkaline phosphatase (ALP) positive colonies after 2–3 weeks of culture [6]. Animal studies have shown that intravenous (IV) administration of bFGF to young and aged

ovariectomized (OVX) rats, an established model for human post-menopausal osteopenia, resulted in increased bone formation with significantly increased osteoblast surface, osteoid surface, and osteoid volume compared to that of the control rats [7-9]. bFGF has been found to stimulate the uncommitted BMSC to differentiate into osteoblasts *in situ,* and to form new osteoid tissue both in normal and OVX rats [7,10]. The bone apposition effects of bFGF can be observed as early as 24 h after treatment in OVX rats, with the osteoblast surface, osteoid surface, and the osteoid volume increasing to more than double as compared to the baseline group [11]. These bone anabolic properties of bFGF, make it an attractive therapeutic agent with wide ranging potential for use in osteoporosis therapy. A careful inspection of the reported studies, however, indicates the beneficial effect of bFGF to be manifested after administration of relatively high doses. The bFGF doses utilized ranged between 100 and 200 μg/kg for studies that involved intravenous (IV) injections [7, 11-13]. For subcutaneous (SC) injection studies, even higher doses (1000 μg/kg) had to be used [14-17]. bFGF is a pluripotent cytokine, and administration of this protein in such high doses is expected to display non-specific effects at other sites. Indeed, adverse effects such as moderate to severe anemia, and glomerular hypertrophy were recently reported [18], and these extra-skeletal effects could restrict its use in osteoporosis therapy. These observations emphasize the importance of using lower doses of systemic bFGF to bring about the desired effect on bones, and thereby avoid any adverse effects at extra-skeletal sites. Previous *in vitro* studies from our laboratory have shown that osteogenic stimulation of BMSC was possible even with bFGF concentration as low as ~10 ng/mL, a dose significantly lower than the one

required for the bone morphogenetic proteins (BMPs), such as BMP-2 that required ~500 ng/mL for an effective stimulation of mineralization [19, 20].

In this study, we explored the bone anabolic affects of bFGF at a dose significantly lower (25 μg/kg) than the ones reported in the literature. Normal rats were utilized to investigate the anabolic effect of bFGF, as well as OVX rats to determine the effect of estrogen depletion on the bFGF response. BMSC was harvested from the treated animals and examined the osteogenic markers of BMSC in culture, since no other studies have probed the changes in this cell population. Changes in cell proliferation, ALP activity, mineralization and expression of extracellular matrix proteins were evaluated. In parallel, changes in bone mineral density (BMD) were also assessed. Our results showed that low dose of bFGF depleted the osteogenic population in bone marrow and increased BMD in OVX rats, which is consistent with the expected anabolic effect of the protein in the bone microenvironment.

5.2 Materials and Methods

5.2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Hank's Balanced Salt Solution (HBSS; without phenol red), penicillin/streptomycin (10,000 U/mL/10,000 μg/mL solution) were from GIBCO (Grand Island, NY). The heat-inactivated fetal bovine serum (FBS) was from Atlanta Biologics (Atlanta, GA). All tissue culture plastic ware was from Corning (Corning, NY). Recombinant bFGF (1 mg/vial, containing 50 μg of bovine serum albumin per 1 μg of bFGF) was manufactured by R&D systems (Minneapolis,

MN) and it was obtained from the NCI Biological Resources Repository (Bethesda, MD). A CyQUANT cell proliferation kit from Molecular Probes (Portland, OR) was used to quantitate DNA concentration in cell lysates. The tissue culture reagents dexamethasone (Dex), ß-glycerol phosphate (ß-GP), ascorbic acid, the ALP substrate p-nitrophenol phosphate (p-NPP), and p-nitrophenol (p-NP) were from Sigma (St. Louis, MO) and they were used without further purification.

5.2.2 Animal Care and Experimental Design

Ten normal and ten ovariectomized female Sprague-Dawley rats were purchased from Charles River Laboratories (St. Laurent, QC, Canada). Two-month old rats were shipped to the University of Alberta (Edmonton, Canada) and the animals were housed for an additional five months to ensure development of osteopenia in the OVX rats. While being maintained in pairs in sterilized cages on a 12:12 h light:dark cycle, the rats were provided with standard commercial rat chow and tap water *ad libitum*. All the animal procedures were performed according to the guidelines of the Canadian Council on Animal Care, and were approved by the Animal Welfare Committee at the University of Alberta.

The rats were randomly assigned into four groups (5 rats/group) depending on the type of animal and treatment received: normal-saline, normal-bFGF, OVX-saline and OVX-bFGF. The rats were injected in their tail veins with saline (300 µL), or bFGF dissolved in saline (10 µg in 300 µL) 6 times a week (daily) for two weeks. Twenty four hours after the final injection, all the rats were sacrificed by asphyxiation in a CO_2 chamber and weighed. Systemic estrogen depletion was confirmed in the case of all the

OVX rats not only by visual inspection of the uterus, where significant atrophy was observed, but also from the reduced uterus weight of the OVX rats compared to the normal ones (0.155 ± 0.061 g vs. 0.815 ± 0.286 g, respectively; $p<0.001$). Both femora and tibiae of the rats were harvested, and the femora was used for BMSC isolation while the tibiae were stored at -20°C for further analysis of mineral density.

5.2.3 Cell Isolation and Culture

The BMSC were isolated aseptically from both femurs. The proximal and distal ends of the bones were cut and the whole bone marrow was flushed out from the diaphyses with approximately 15 mL of basal medium (DMEM, 10% FBS, 50 mg/L ascorbic acid, 100 U/mL penicillin, and 100 μg/L of streptomycin) using a syringe with a 23G needle. The cells were pooled to obtain a single cell suspension and were centrifuged at 600 g for 10 min. The cell pellet was resuspended in fresh basal medium and subsequently the cell density was determined with a hemacytometer. The BMSC were seeded in multiwell plates in basal medium and were used for different assays at pre-determined time points. The plates were placed in a humidified incubator with 5% CO_2 atmosphere at 37°C. The cells were allowed to attach for four days, after which the non-adherent cells were discarded. The medium was then changed to osteogenic medium (the basal media with 10^{-8} M Dex and 3 mM ß-GP) to support mineralization. The osteogenic medium was subsequently replaced once a week. The concentrations of Dex and ß-GP used in the osteogenic medium were optimized prior to this study [20, Appendix C].

5.2.4 Alkaline Phosphatase (ALP) Assay

ALP is a membrane-bound enzyme that serves as an early marker for osteoblast differentiation, whose activity typically peaks prior to the onset of mineralization [21]. In order to determine the ALP activity, the BMSC in 6-well plates (in triplicate) were washed with HBSS twice and lysed with 300 μL ALP buffer (0.5 M 2-amino-2-methylpropan-1-ol and 0.1% (v/v) Triton-X; pH 10.5). Two hours after lysis, 150 μL of the lysis solution was aliquoted to the wells of a 48-well plates, into which 150 μL of 1 mg/mL ALP substrate p-NPP was added to give a final concentration of 0.5 mg/mL p-NPP. The ALP activity was measured spectrophotometrically ($\lambda_{absorbance}$: 405 nm) using a multiwell plate reader (Bio-Tek, Winooski, VT, USA) at 8 regular periodic intervals for up to 12 min, for three different time points of the culture (day 4, 7 and 11). The ALP activity, which was determined in duplicate for each lysate, was reported in terms of the p-NPP product formed (p-NP; in mmol/min/mL), and normalized with the DNA content in each lysate to obtain a specific ALP activity (ALP/DNA).

5.2.5 DNA Assay

To determine the DNA content in the wells, the remaining cell lysis solutions from the ALP assay (day 4, 7 and 11) were frozen at -20°C, thawed at a suitable time, and analyzed with the CyQUANT DNA kit according to the manufacturer's instructions. The assay was performed using a fluorescent plate reader ($\lambda_{excitation}$: 480 nm, $\lambda_{emission}$: 527 nm), and the DNA standard provided with the kit was used for the calibration curve.

5.2.6 Mineralization Assay

The medium in the wells were removed and the wells were then washed with HBSS twice and treated with 1 mL of 0.5 M HCl overnight, to determine the amount of mineralization. Samples from the two time points (week 2 and 4) were stored at 4°C and analyzed together to minimize any variations. Twenty micro liter aliquots of the samples were used to quantify the amount of dissolved calcium by using a spectrophotometric ($\lambda_{absorbance}$: 574 nm) method with a multiwell plate reader [22]. The reference solutions from SIGMA were used to generate the calcium standard curve, and the amount of mineralized calcium was expressed in terms of mg/well.

5.2.7 RT-PCR Analyses for Extracellular Matrix Proteins

BMSC in 6-well plates were harvested using Trizol reagent (Invitrogen) at two time points (day 7 and 14) and stored at -20°C. The total RNA was extracted using the RNeasy Mini Kit (Qiagen, GmbH) following the manufacturer's recommendations. Freshly isolated RNA was quantified fluorometrically ($\lambda_{excitation}$: 480 nm, $\lambda_{emission}$: 527 nm) using the RNA stain SYBR Green II (Cambrex Laboratories, Rockland, ME). 0.3 μg of the isolated RNA was then used for the reverse transcription (RT) reaction using the Omniscript kit (Qiagen, GmbH). The resulting cDNA was used as a template for the Polymerase Chain Reaction (PCR) amplification of the genes of interest namely, osteocalcin (OCN), osteopontin (OPN), bone sialoprotein II (BSP) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as the

internal control. The rat specific primer sequences for OCN and OPN previously reported by Sun et al. [23], and BSP and GAPDH by Shimuzu et al. [24], were used in the 10 nM scale for the amplification reaction. The primers used in this study (Peptide Synthesis Unit, University of Calgary, Calgary, AB) were as follows:

OCN (462 bp)	FP	5'-ATGAGGACCCTCTCTCTGCTCACT-3'
	RP	5'-ACCGTTCCTCATCTGGACTTTA-3'
OPN (941 bp)	FP	5'-CAACCATGAGACTGGCAGTGGTTTGC-3'
	RP	5'-GCCTCTTCTTTAATTGACCTCAGAAG-3'
BSP (73 bp)	FP	5'-TCCTCCTCTGAAACGGTTTCC-3'
	RP	5'-CGAACTATCGCCATCTCCATT-3'
GAPDH (307 bp)	FP	5'-AGATGGTGAAGGTCGGTGTC-3'
	RP	5'-ATTGAACTTGCCGTGGGTAG-3'

(FP - Forward Primer, RP - Reverse Primer)

Linearity of the PCR conditions (i.e., cDNA amounts and number of cycles) was determined in preliminary experiments (data not shown). The optimized parameters for OCN and OPN were 35 cycles, 95°C denaturation for 1 min, 58°C annealing for 1 min and 72°C extension for 10 min. For BSP and GAPDH, the optimized parameters were 40 cycles, 95°C denaturation for 15 s, 64°C annealing for 50 s, and 77°C extension for 12 s. The relative expression of the genes of interest was assessed by densitometric detection of specific molecular weight bands corresponding to the respective PCR amplified products (OCN 462 bp, OPN 941 bp, BSP 73 bp and GAPDH 307 bp). The ethidium bromide-stained 2% agarose gels were photographed with an Alpha Innotech bioimaging system for this purpose. The densitometry values obtained for OCN, OPN and BSP were then normalized with those obtained for GAPDH.

5.2.8 DEXA Analyses

The BMD of the total tibiae was measured by dual-energy X-ray absorptiometry (DEXA) using the Piximus Mouse Densitometer. Individual tibia from each rat was separately sent to Oregon Health Sciences University (Portland, OR) and University of California at Los Angeles (Los Angeles, CA), and the operator was blinded to the treatment received by each of them. BMD (g/cm^2) of each tibia was calculated by dividing the bone mineral content (g) by the projected bone area (cm^2).

5.2.9 Statistical Analyses

All of the data generated is expressed as mean ± standard deviation (SD). For comparison of the variables among the treatment groups, the significance of the main effects were tested using single factor ANOVA and paired *t*-tests assuming equal variance ($p<0.05$).

5.3 Results

5.3.1 Cell Harvest and Proliferation in Culture

The recovery of cells from the femurs of the bFGF-treated normal and OVX rats were $133 \pm 54 \times 10^6$ (n=5) and $119 \pm 34 \times 10^6$ (n=5) cells, respectively. The cell recovery for the saline-treated normal and OVX rats were $164 \pm 47 \times 10^6$ (n=5) and $163 \pm 46 \times 10^6$ (n=5) cells, respectively. The cell counts obtained from the rats treated with bFGF was

lower as compared to the rats injected with saline for both normal and OVX rats (Fig. 3-1). The differences, however, were not statistically significant for each type of rat.

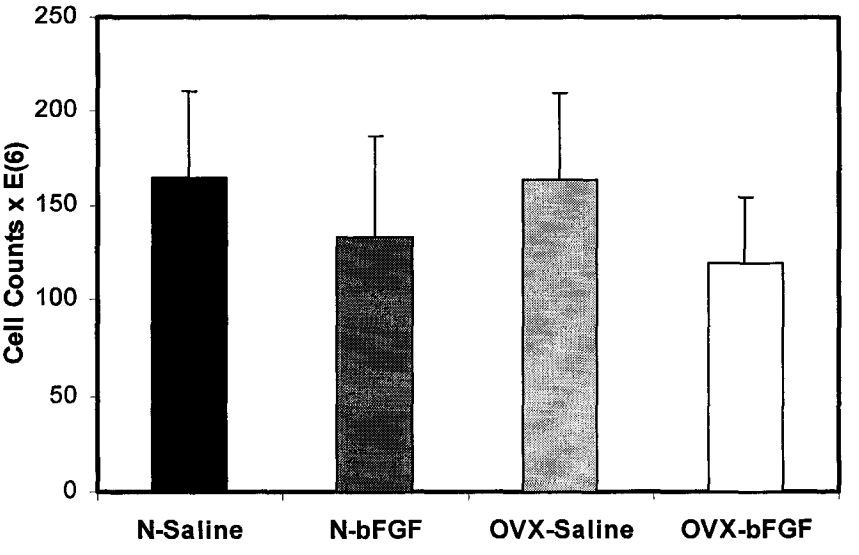

Figure 3-1. Effect of bFGF treatment on cell counts harvested from femurs of rats. The BMSC were isolated aseptically from the rats belonging to the different treatment groups, and the total number of cells recovered was counted for each rat using a hemacytometer. Each bar represents mean ± SD number of cells recovered from femurs of each treatment group (n=5 rats per group).

To assess whether bFGF treatment had an effect on proliferation of BMSC in culture, the DNA content of the wells was measured at pre-determined time points (Fig. 3-2). On day 4, all of the treatment groups were found to have similar amounts of DNA/well. On day 7, the DNA content/well were considerably lower (~50% of day 4) for the bFGF-treated normal and OVX rats, while the DNA content/well was comparable to the previous time point for the control groups. On day 11, the trend observed for day 7 continued, the only difference was that the DNA content/well for saline-injected groups almost doubled in comparison to day 7, while a relatively small increase was observed in the bFGF-treated groups. The lower amounts of DNA observed for the bFGF-treated rats suggested reduced proliferation of BMSC *in vitro*.

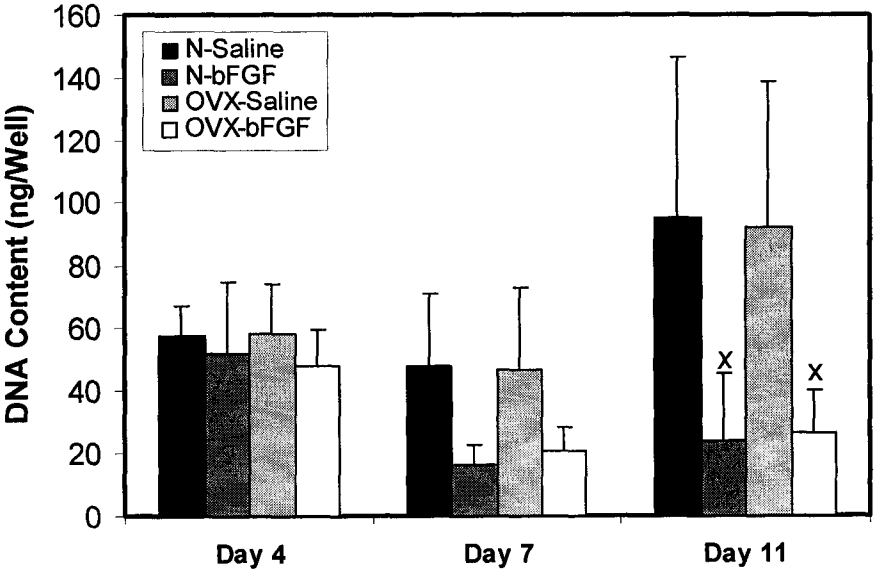

Figure 3-2. Effect of bFGF treatment on DNA content of BMSC at different time points in culture. BMSC were seeded in 6-well plates with basic medium (in triplicate), and allowed to attach for four days. The non-adherent cells were then discarded, and the medium was replaced with osteogenic medium. The cells were lysed with ALP buffer and DNA content/well was assessed (in duplicate) at pre-determined time points. The results are summarized as the mean ± SD DNA amount/well for each treatment group (n=5 rats per group). x : $p<0.05$ compared to saline-treated normal or OVX rats.

5.3.2 Specific ALP Activity

The specific ALP activity in the bFGF treated groups was similar to the control groups initially (day 4), but it was considerably lower subsequently on days 7 and 11 (Fig. 3-3). A progressive increase in the levels of ALP was observed for the saline-treated rats. For the bFGF-treatment groups, the specific ALP activity remained low during the 11-day evaluation period. No differences in the specific ALP activity were apparent between the normal and OVX rats at all the 3 time points (whether saline or bFGF treated).

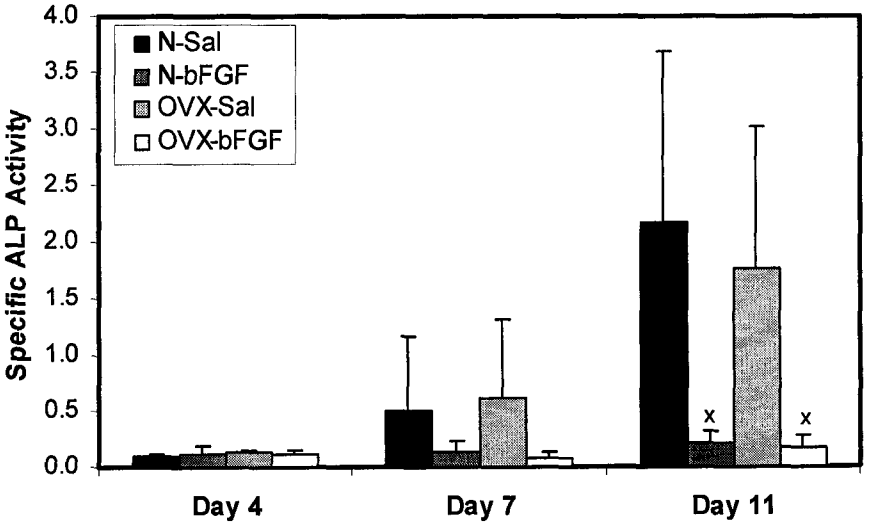

Figure 3-3. Effect of bFGF treatment on specific ALP activity of BMSC at different time points in culture. BMSC were seeded in 6-well plates with basic medium (in triplicate), and allowed to attach for four days. The non-adherent cells were then discarded, and the medium was replaced with osteogenic medium. The cells were lysed with ALP buffer and ALP Activity was assessed (in duplicate) at pre-determined time points. ALP activity was reported in terms of the p-NPP product formed (p-NP; in mmol/min/mL), and normalized with the DNA content in each lysate to obtain the specific ALP activity (ALP/DNA). The results are summarized as the mean ± SD specific ALP activity for each treatment group (n=5 rats per group). x : $p<0.05$ compared to saline-treated normal or OVX rats.

5.3.3 Extent of Mineralization

The extent of mineralization was similar for all study groups at the initial (week 2) time point (Fig. 3-4). By week 4, the extent of mineralization increased in all groups, but the mineral apposition in the bFGF-treated groups of normal and OVX rats was 2.5 fold and 1.9 fold lower than the control groups, respectively. No significant differences were observed between the bFGF-treated normal and OVX rats at week 2 and week 4.

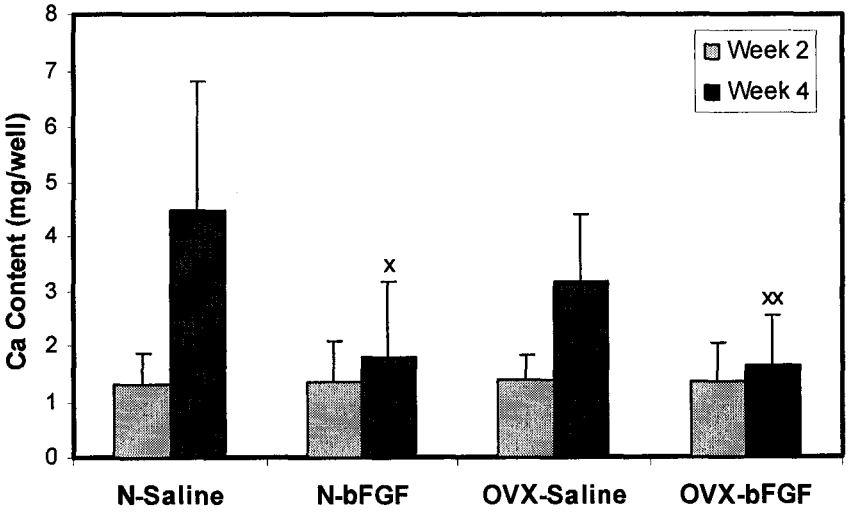

Figure 3-4. Effect of bFGF treatment on mineralization of BMSC in culture. The BMSC were seeded in basic medium, allowed to attach for four days, after which the medium was changed to an osteogenic medium. The cells were cultured for a period of four weeks, where the mineralization was assayed at the end of two and four weeks. Each bar represents mean ± SD calcium content/well for each treatment group after 2 and 4 weeks in culture (n=5 rats per group). Each bar represents the mean ± SD (n=5 rats). x: $p<0.05$ for bFGF- vs. saline-treated normal rats (week 4). xx: $p<0.05$ for bFGF- vs. saline-treated OVX rats (week 4).

5.3.4 Expression of BSP, OCN and OPN by BMSC

The RT-PCR analyses at week 1 revealed that bFGF stimulated the expression of all three extracellular matrix genes (BSP, OCN and OPN) in BMSC from normal and OVX rats to different levels (Fig. 3-5A, B). The highest stimulation was observed for OPN for both normal and OVX rats ($p<0.05$ for bFGF-treated vs saline-injected rats). There was a general trend for upregulation of BSP and OCN for the bFGF-treated rats (both normal and OVX rats), but these changes were not as significant as the changes in the OPN expression. By day 14, the expression levels of the three markers were significantly reduced, with the bFGF-treated groups showing elevated expression of OPN, but not at a significantly higher level.

A

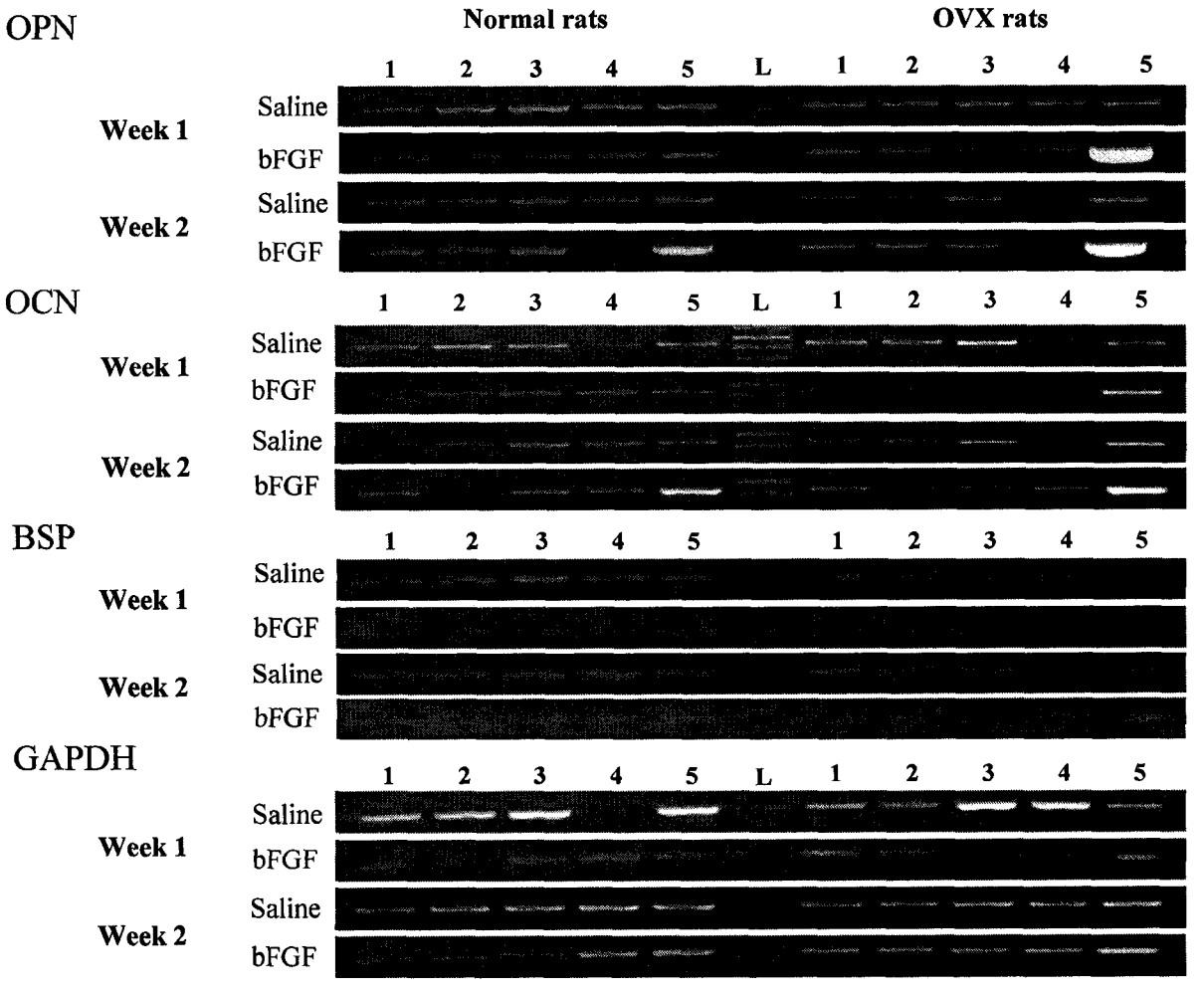

Figure 3-5A. The effect of bFGF treatment on expression of OPN, OCN, and BSP in BMSC as analyzed by RT-PCR. The mRNA was isolated from the cultures at the end of week 1 and week 2, reverse transcribed and probed using specific primers for the genes of interest. The cDNA was amplified and the products were run on 2% agarose gels for densitometric analysis. The cDNA levels of OPN, OCN, BSP and GAPDH for individual rats (numbered 1 to 5) in each treatment group. cDNA was from BMSC maintained in culture for 1 and 2 weeks. Lane L contained 1 kb-molecular weight ladder.

B

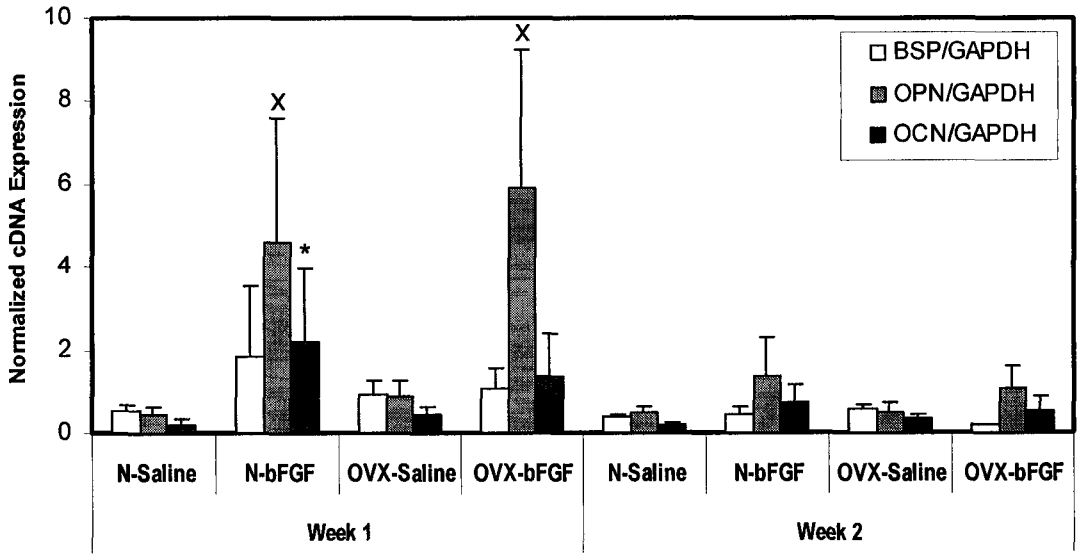

Figure 3-5B. Densitometric analyses of OPN, OCN and BSP expression at week 1 and week 2 after normalization with GAPDH. Significant differences were observed for (a) OPN between bFGF- and saline-treated normal rats on week 1 (x : $p<0.005$), (b) OPN between bFGF- and saline-treated OVX rats on week 1 (x : $p<0.005$), and (c) OCN between bFGF- and saline-treated normal rats on week 1 (x : $p<0.05$).

5.3.5 Bone Mineral Density of Tibia

The tibiae from saline-treated normal rats had a higher total BMD than the saline-treated OVX rats (Fig. 3-6A, 3-6B; $p<0.05$), confirming the development of osteopenia after the ovariectomy. The bFGF treatment reduced the BMD values for normal rats in comparison to the saline-treated normal rats, although the difference was not significantly different ($p>0.05$). On the other hand, the bFGF treatment in OVX rats significantly increased the BMD values ($p<0.05$) compared to the saline-treated OVX rats.

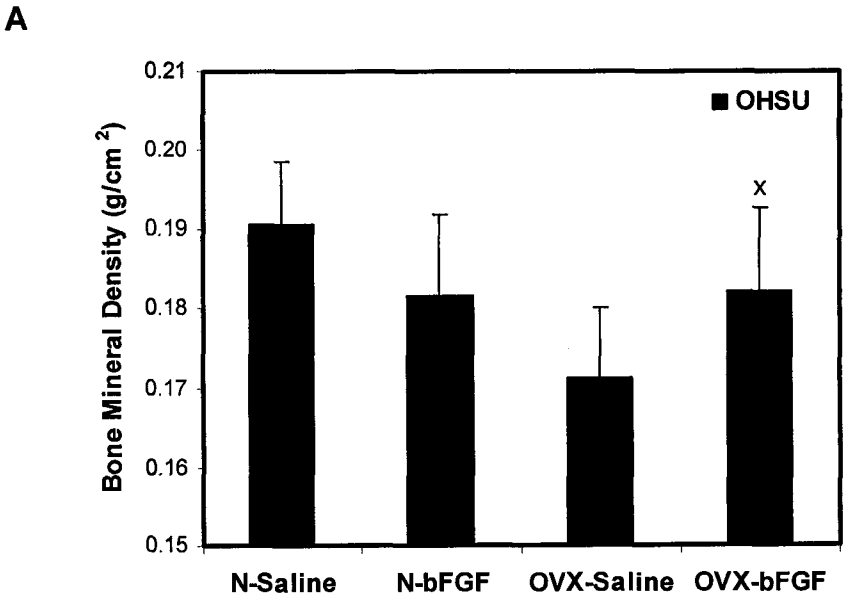

Figure 3-6A. The effect of bFGF treatment on BMD of tibiae as measured by using DEXA (PIXImus) at OHSU. Each bar represents the mean ± SD BMD for each treatment group (n=5 rats per group).

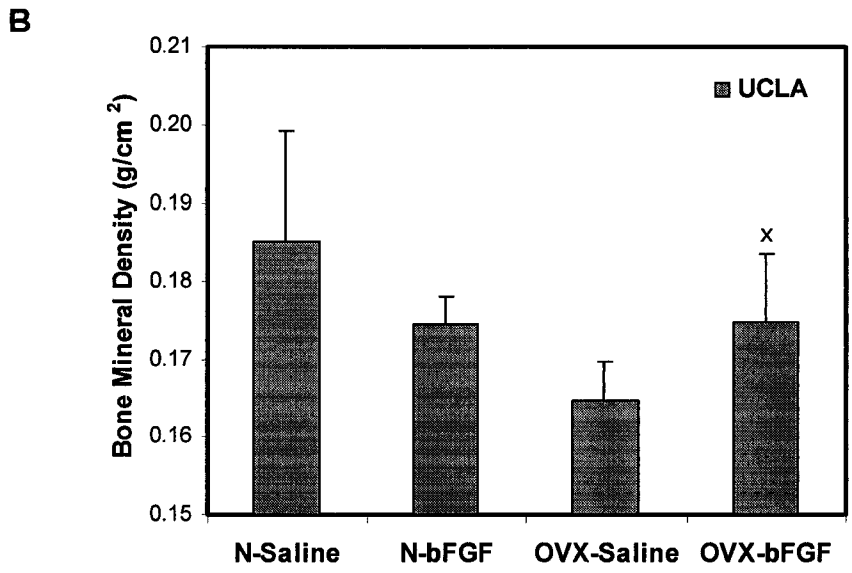

Figure 3-6B. The effect of bFGF treatment on BMD of tibiae as measured by using DEXA (PIXImus) at UCLA. Each bar represents the mean ± SD BMD for each treatment group (n=5 rats per group). X ; $p<0.005$ between saline- and bFGF-treated OVX rats.

5.4 Discussion

The present study was performed to further understand the influence of bFGF administration on systemic bone formation. More specifically, changes in the osteogenic cell population in bone marrow were monitored *in vitro* after systemic treatment with a low dose of bFGF. The dosage of bFGF used in this study was based on an in-house bFGF pharmacokinetics assessment (Appendix B) and *in vitro* cell culture studies, where dose-response curves for bFGF effect on osteogenic properties of rat BMSC were generated (Appendix C). When the rat BMSC in our culture studies were exposed to, low concentrations of bFGF (2-10 ng/mL), they exhibited stimulation of ALP activity and mineralization [20]. Short duration treatment (1 week) at higher doses of bFGF (50 ng/mL) was also stimulatory for osteogenesis in older rats (~7 months), however, such a high dose for a longer duration (>3 weeks) inhibited osteogenic activity of the BMSC [20]. The bFGF dose administered in this study (25 μg/kg x 6 days/week x 2 weeks) was chosen to yield a concentration of ~10 ng/mL at the tibiae, and was significantly lower than other similar studies found in the literature that explored the anabolic effects of bFGF. The bFGF doses used in other previous studies were 100-200 μg/kg x 7 days/week, for 1 or 2 weeks of IV injection [7,11-13], or 1000 μg/kg x 5 days/week for 8 weeks [14-17] of SC injection. Our rationale for using lower doses than those reported in the literature was based on the concern about the possible undesirable effects of the bFGF at high doses. Despite the observed anabolic effects of bFGF *in situ*, no studies have reported the changes, if any, in the bone marrow cell population.

bFGF is well known to act as a mitogen on BMSC in culture, stimulating proliferation and growth of the explanted cells [20,25-28]. Previous cell culture studies

from the authors' lab indicated at least 2-fold increased cell numbers as a result of bFGF treatment of BMSC, which was consistent with others who reported as much as 2-3 fold increased cell growth/proliferation in *in vitro* studies [26,27]. Unlike our expectations, increased cell numbers were not obtained from the marrow of bFGF-treated rats, but on the contrary, a general trend of lower cell counts was observed (note that this decrease did not reach to a significant level). Recovery of higher numbers of cells was expected if bFGF had exhibited a mitogenic effect in the bone marrow environment. The attachment of the bone marrow cells to the tissue culture plastic was not affected whether the cells were exposed to bFGF or not (based on the DNA content/well from day 4 cultures; Fig. 3-2). However, there was no evidence of any mitogenic effect of bFGF on the attached BMSC, resulting in lower cells numbers (based on the DNA contents) during the *in vitro* culture period, as compared to BMSC cultures from saline-treated rats.

The osteoblastic activity in BMSC cultures was assessed based on specific ALP activity, mineralization, as well as expression of select extracellular matrix proteins. The specific ALP activity of the attached BMSC was low initially (day 4), and equivalent for all groups whether the cells were obtained from normal or OVX rats. Subsequently, however, BMSC from bFGF-treated rats exhibited a lack of ALP induction, unlike the BMSC from saline-treated rats that had sustained levels of ALP activity after 11 days. During this time period, expression of OPN was particularly elevated (week 1) for BMSC from bFGF-treated rats, but was subsequently down-regulated to lower levels. The up-regulation of OPN expression observed in this study was consistent with other previous studies done on (i) bFGF-treated human BMSC after 20 days in culture (5 ng/mL, [29]), and (ii) rat BMSC treated with bFGF in an osteogenic medium (10 ng/mL, [30]) in three-

dimensional matrices. The OPN expression was typically the most sensitive marker among the common matrix proteins used in these studies. The effect of bFGF treatment on OCN expression in the current study depended on the type of rat (normal vs. OVX) used. Contrary to the absence of bFGF effect on BMSC from OVX rats observed here, Power et al. (2002) reported a higher level of OCN expression from tissues of the vertebral bodies of aged OVX rats after high-dose of bFGF treatment [12]. Their study might better reflect the bFGF effect *in situ*, due to the possibility that alterations in specific gene expression might have occurred during the *in vitro* explant culture in our study. Collectively, our results from extracellular matrix synthesis and ALP expression exhibited some inconsistency, i.e., specific ALP activity (early marker of osteoblast differentiation) was reduced but OPN expression (late marker of osteoblast differentiation) was increased. However, mineralization in cultures was similar to the ALP activity results, with the BMSC derived from bFGF-treated rats exhibiting lower mineralization at the end of the culture period.

To the best of our knowledge, this is the first study to report DEXA analyses of bFGF induced bone deposition in an animal model. DEXA analyses was performed on the tibiae mainly because the impact of ovariectomy-induced bone loss and the subsequent recovery from it following bFGF therapy would be more evident on a long bone, as compared to other skeletal tissues. Previous studies exploring anabolic effects of bFGF on bone commonly relied on histological assessment of changes in *in situ* cellular activities [16, 17]. Tissue-level changes were examined in two previous studies utilizing micro-computed tomography (micro-CT or μCT), which reported increased trabecular thickness and connectivity in OVX rats as a result of bFGF treatment [16, 17].

This is indicative of sufficient anabolic effect of bFGF resulting in the observed tissue–level changes in mineralized bone mass. The DEXA results obtained in this study also indicated that the low dose of bFGF was sufficient to provide a tissue-level increase in mineral density in OVX rats. Four months after ovariectomy, it is expected that as much as ~80% loss of the trabecular structure in the tibia would be exhibited [17], facilitating any anabolic effect of bFGF to be more readily detectable in OVX rats. The increased tibial BMD observed for the OVX rats supported our *in vitro* culture data that bFGF treatment hastened osteogenic differentiation in the marrow, depleting the osteogenic precursors from the marrow. Our results also indicated that the depleted pool of progenitor cells was not replenished during the 2-week bFGF administration period. In addition to bone marrow cells, the bone lining cells could also contribute to mineral apposition in OVX rats (as previously reported in the case of bFGF response of OVX rats [11]), however, the explant cultures will not contain the bone lining cells since they are unlikely to be extracted.

Unlike the case of OVX rats, no beneficial effect of bFGF was seen on BMD of tibiae from the normal rats. One reason for this observation might have been the higher initial tibia BMD in normal rats, so that the incremental effect of bFGF was not readily detectable with DEXA. Perhaps a more sensitive technique, such as micro-CT, might have been more useful in this respect. A second reason might be the intrinsic differences in bFGF responsiveness of BMSC from the two types of rats. bFGF at a relatively high dose (50 ng/mL) gave differential effects on osteogenic differentiation of BMSC *in vitro*, depending on whether the cells were obtained from normal or OVX rats [25]. For example, bFGF suppressed the ALP activity of BMSC from normal, but not OVX rats,

while BMSC mineralization was unaffected for cells from normal rats, but was suppressed for cells from OVX rats [25]. Whether such an intrinsic differential response was also present *in situ* remains to be determined. Among the published literature, no report has compared the bFGF response in normal vs. OVX rats in a head-to-head study. However, separate studies did indicate the desired anabolic effect of bFGF in both normal [4,5], and OVX rats [7-17]. The normal rats used in these studies, however, were relatively young (6-8 weeks at study onset) unlike our rats that were ~30 weeks, and also were of different species than the ones used in this study. bFGF is known to act differently on young and aged rats [27], and the age differences in normal rats might explain the lack of anabolic effect observed in this study. Also, since we are employing even a lower dose of bFGF than the other studies with normal rats, it is possible that we are simply not imparting a strong anabolic effect to be detected by DEXA. The other possibility might be that bFGF is stimulating osteoclastic activity in the normal rats, such that any anabolic effect induced would be dominated by a stronger osteoclastic activity. *In vitro* studies using bone marrow cells have indicated mostly a stimulatory role of bFGF on osteoclastogenesis and osteolytic activity [31-33], with some exceptions [34]. In OVX rats, systemic bFGF did not affect osteoclastic activity [8, 12, 15], or decreased it in some studies [9, 11, 17], but no information exists as to whether such an effect holds true for normal aged rats. Further studies on this issue will be required in order to clarify whether bFGF stimulates osteoclast activity in normal rats, obliterating the anabolic effect of bFGF.

In summary, the results of this study confirmed the beneficial effect of systemic bFGF in stimulating deposition of new mineralized tissue in the long bones of OVX rats

after only two weeks of treatment. Such an anabolic effect was not detectable in the age-matched normal rats. The cells recovered from the bone marrow of bFGF-treated rats exhibited a lower extent of osteogenic cells, possibly reflecting the effect of enhanced osteogenic differentiation *in vivo* under the influence of bFGF. The dose of bFGF used in this study was lower than the previously reported studies, indicating the possibility of reducing the administration dose of this powerful anabolic agent, and thus its side-effects at other sites while preserving its bone anabolic effects. The minimum effective dose (i.e., dose lower than the 25 μg/kg used here) will need to be identified in future studies. Further investigations will also be required to understand the differences between bFGF response of normal and OVX rats *in situ*. The ability to obtain an osteogenic response at low administration dose of bFGF is encouraging to further explore the potential of this anabolic agent in osteoporosis therapy.

5.5 References

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CONCLUSIONS AND FUTURE AVENUES

Our *in vitro* culture studies showed that both bFGF and BMP-2 were osteogenic and were capable of stimulating mineralization under select set of culture conditions, but significant differences in their effects were evident. Irrespective of the duration of exposure, a clear dose-response was consistently obtained for BMP-2, with progressively higher levels of mineralization upto 500 ng/mL. Interestingly, although bFGF effects were evident even at ~ 10 ng/mL, on continuous treatment, no significant effects on short-time exposure was observed. The extent of mineralization of BMC varied with the source of the cells, it being significantly lower for old rats than for young rats. Interestingly, a synergistic effect of lower doses of BMP-2 on bFGF responses was observed on BMC from the old rats, but not from the young rats. The BMC from older rats is more representative of osteoporosis patients, and this result indicates the possibility of utilizing the synergistic effects of bFGF and BMP-2 in a clinical scenario. From our studies we could conclude that, although bFGF was more potent for stimulating osteogenic parameters, the BMP-2 effects were more lasting on the BMC.

Soluble factors such as hormones and other peptide-based regulators that may modulate the cellular responses to bFGF and BMP-2 exposure were not incorporated into our *in vitro* cultures. Further studies will be required to differentiate whether bFGF might inhibit the osteogenic activity of differentiated osteoblasts at higher concentrations or whether it also increases the population of other lineage cells at higher concentrations since multiple cell lineages share the same pluripotent precursor cells as the osteoblasts in the bone marrow environment. In future studies new bone deposition could be assessed using colony formation or nodule assays and additionally by assaying for osteoblast-specific markers to differentiate between these possibilities. Another variable that should

be considered in future studies is the three-dimensional nature of the cellular environment *in vivo*. It was recently recognized that BMC grown within a 3-dimensional configuration displays a differential response to growth factors, such as BMP-2 and Transforming Growth Factor-β, as compared to the cells grown on two-dimensional surfaces. Keeping this in perspective, the results reported in our culture system should be considered as a first line approximation to the possible *in vivo* effects of the growth factor response.

The results of our animal studies confirmed the beneficial effect of low dose systemic bFGF in stimulating deposition of new mineralized tissue in the long bones of OVX rats after only two weeks of treatment. We conclude that in culture, the osteogenic activity of the BMSC derived from the bFGF treated rats was low possibly because more cells were already committed to new bone deposition under the influence of bFGF, as seen from the increased bone mass in the OVX rats. So this possibly leaves the harvested cells deficient in the osteoprogenitor cells. The significantly lower dose of systemic bFGF used in this study compared to previously reported studies, demonstrates the potential of reducing the administration dose of this potent bone anabolic agent, and thus its adverse extra-skeletal effects.

In future studies the minimum effective dose lower than that used in our study needs to be identified. Additional investigations will also be required to gain further insight into the differences between the bFGF response of normal and OVX rats *in situ*. Furthermore in future studies it would also be interesting to assess the osteoclast activity in normal and OVX rats in response to systemic bFGF treatment using techniques such as osteoclast-specific ELISA assays and pit resorption assays. This would give us further insight into how bFGF treatment favours positive bone balance during the bone

remodelling process. Another important area of investigation would be to assess the changes in marrow composition in older rats in response to bFGF treatment, since recent findings suggest that high adipocyte count in bone marrow is directly related to bone loss, as fat cells replace osteoblasts. Future studies should be directed aimed at targeting bFGF to the bones such that the adverse effects at extra-skeletal sites could be minimized.

The promising results obtained from our studies serves as a foundation for numerous additional studies to further expand upon our knowledge about the bone anabolic effects of bFGF and to explore its use in osteoporosis therapy.

APPENDICES[4]

[4] The contents of Appendix B have been submitted to *Tissue Engineering* (2006) as: Varkey M, Kucharski C, Doschak MR, Winn SR, Brochmann EJ, Murray S, Matyas JR, Zernicke RF, and Uludağ H. Osteogenic response of bone marrow stromal cells from normal and ovariectomized rats to low dose bFGF treatment. The contents of Appendix A, Appendix C and Appendix D are unpublished results from Varkey M, Kucharski C, Lin, X and Uludağ H.

7.1 APPENDIX A

Objective:

Preliminary experiment to assess the response of BMSC from normal female Sprague-Dawley rats which were administered systemic bFGF and BMP-2 treatment individually, and in combination.

Bioactivity Model:

1. The rats were administered the appropriate growth factor injection and maintained in pairs in cages for two weeks before sacrifice. The rats were classified into the following treatment and control groups with 3 rats/group: pre-treatment, no treatment, saline, BMP-2, bFGF and BMP-2+bFGF. BMSC was isolated from the femurs of the rats, of which half were seeded in triplicate in dishes for colony formation assays while the other half were seeded in multi-well plates for biochemical assays.
2. Cell viability was assessed on day6 by MTT assay, while on day 6, 14, 21 and 28 ALP activity and DNA content were determined using the cells seeded in the multi-well plates. In the dishes the total number of colonies formed and the ALP positive colonies were counted on day 6 and day 13, while on day 21 the mineralized colonies were counted.

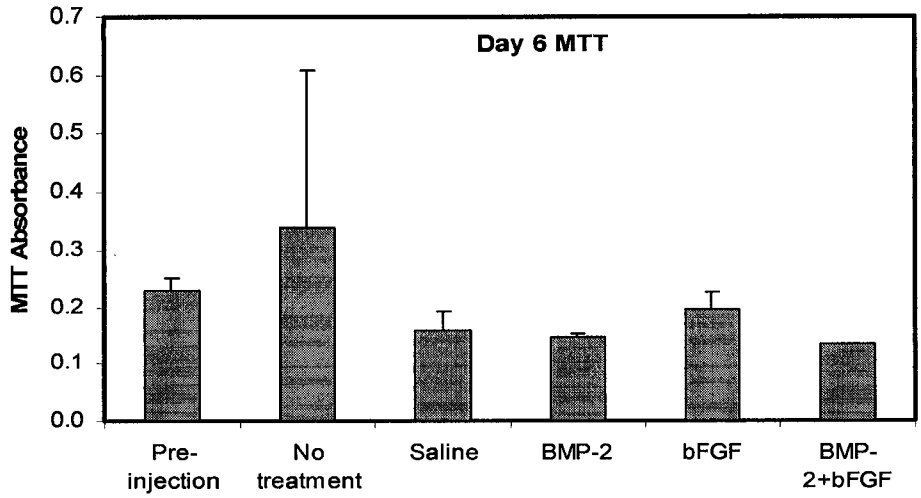

Figure A-1. Rat BMSC cell viability (day 6) in response to systemic GF treatment.

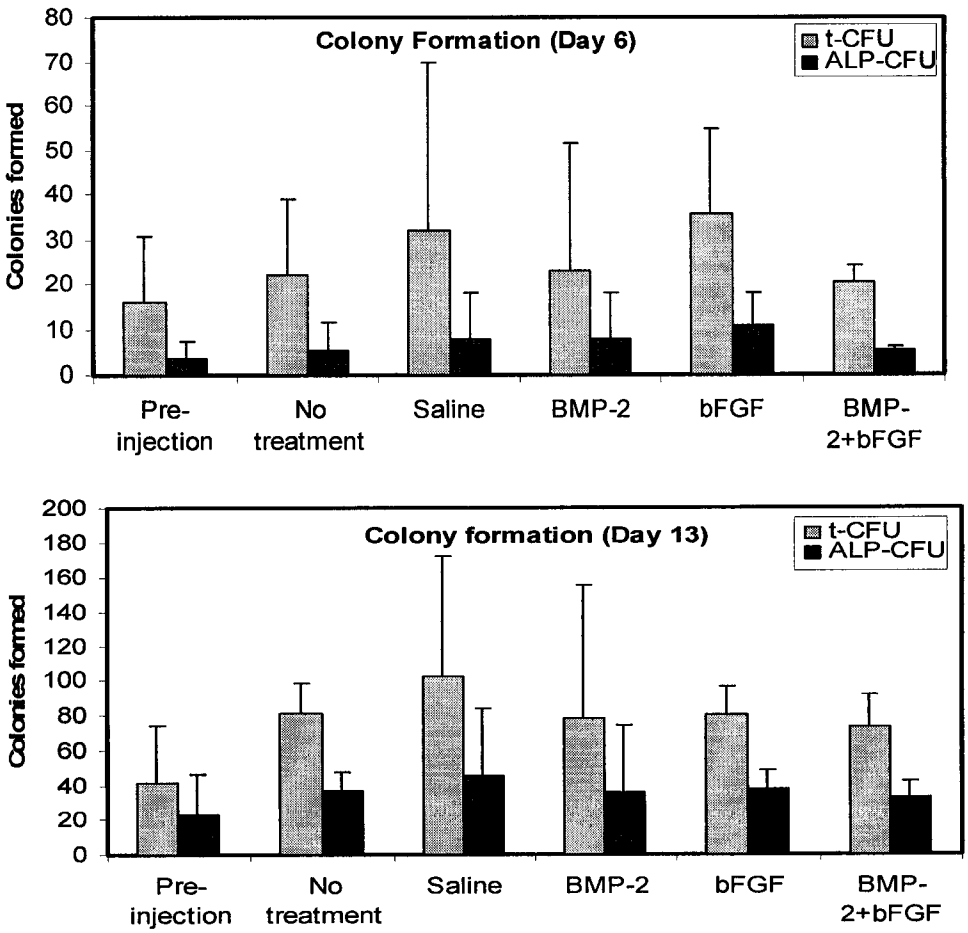

Figure A-2. Colony formation assays on day 6 and 13 using BMSC from rats administered systemic GF treatment.

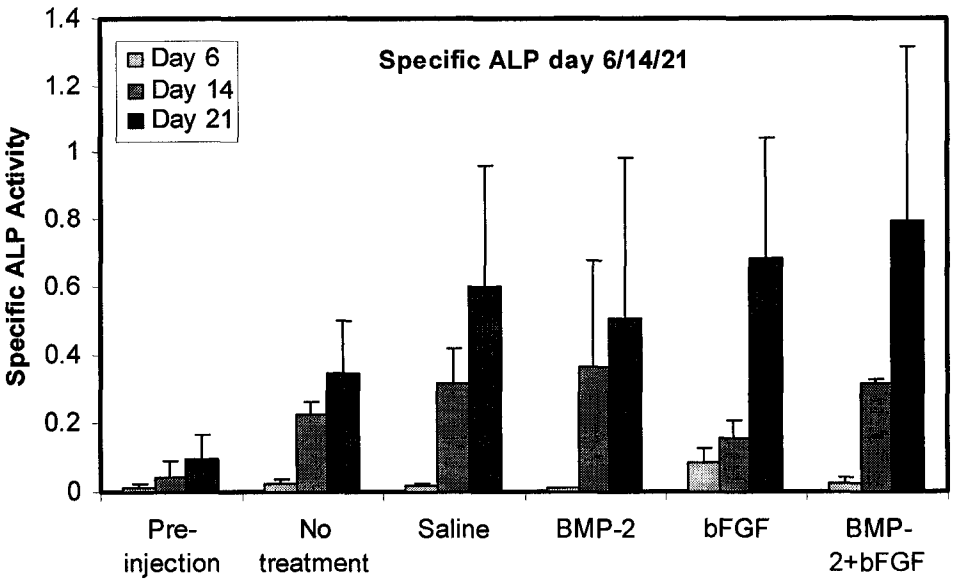

Figure A-3. Specific ALP activity of rat BMSC in response to systemic GF treatment on days 6, 14 and 21.

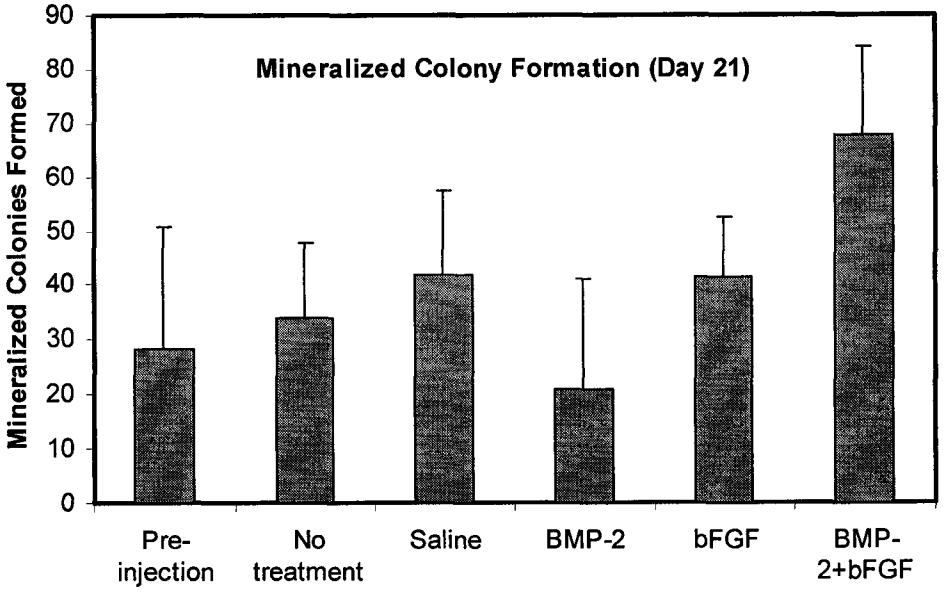

Figure A-4. Formation of mineralized colonies after 3 weeks of culture of BMSC obtained from rats systemically treated with GF.

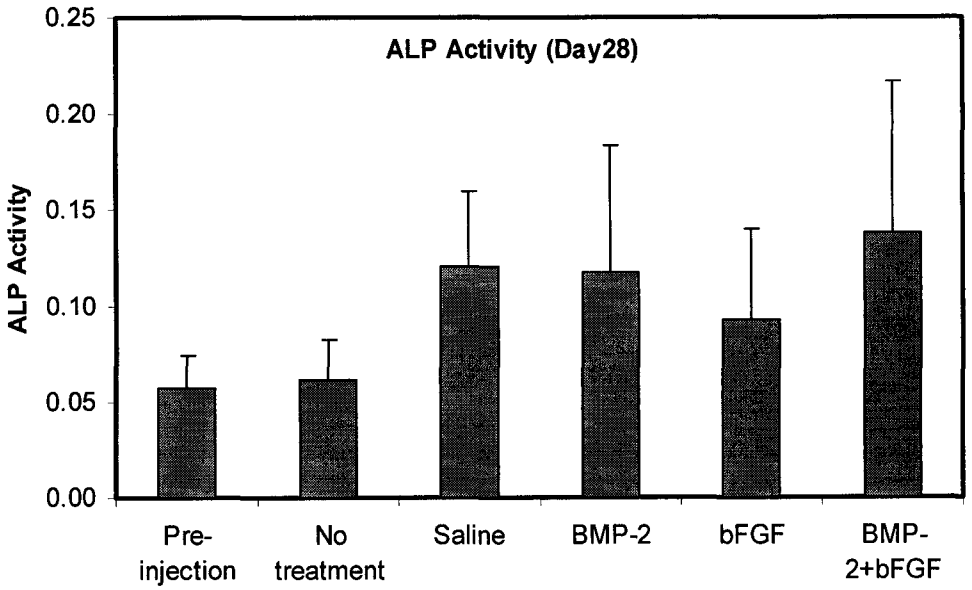

Figure A-5. ALP activity after 4 weeks of culture of BMSC obtained from rats systemically treated with GF.

7.2 APPENDIX B

Objective:

Biodistribution study to measure the amount of bFGF and BMP-2 deposited at bones after systemic administration [1].

Bioactivity Model:

bFGF and BMP-2 were labelled with ^{125}I according to the methods described in Gittens *et al.* 2005 [2] and intravenously injected into rats (n=3) as a 300 μL solution in saline. After 3, 24 and 96 hours, various organs (as indicated in the Figure below) were excised and the amount of radioactivity in each was determined with a γ-counter. The radioactive counts in each organ were normalized with the originally administered counts (except liver and blood, where the counts were further normalized per g of tissue excised). The results are summarized as mean ± SD (Figure B-1, Figure B-2). Note that 0.04-0.05% of administered dose as deposited at femurs, and 0.03-0.04% of the dose at the tibiae. Based on this, the initial injection of 25 μg was expected to deposit 10-12.5 ng and 7.5-10 ng per femur and tibia, respectively.

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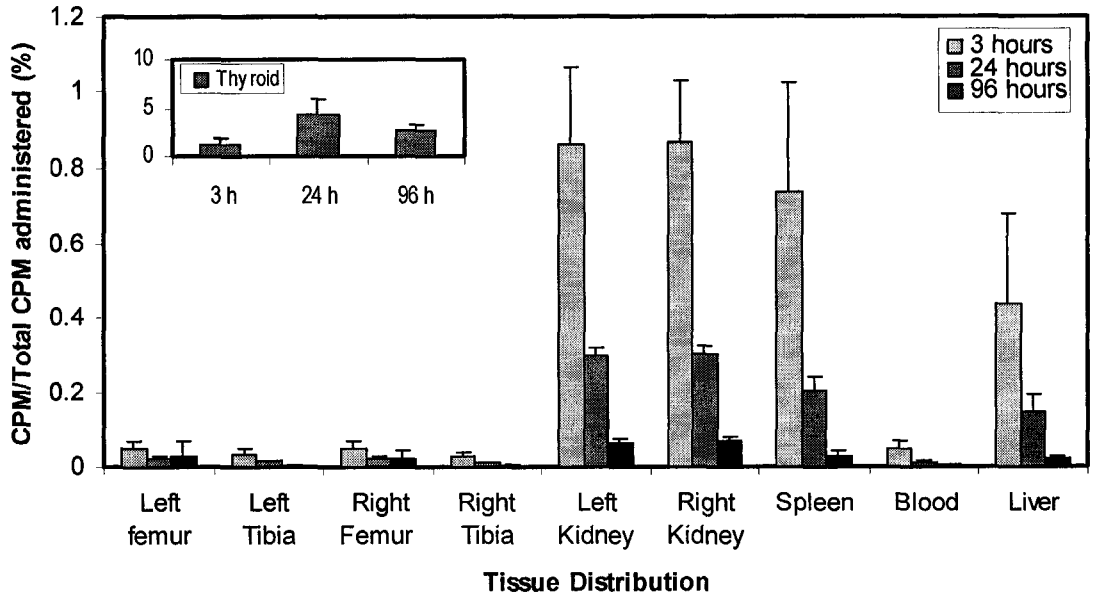


Figure B-1. Pharmacokinetics assessment for bFGF after 3, 24 and 96 h after bFGF injection.

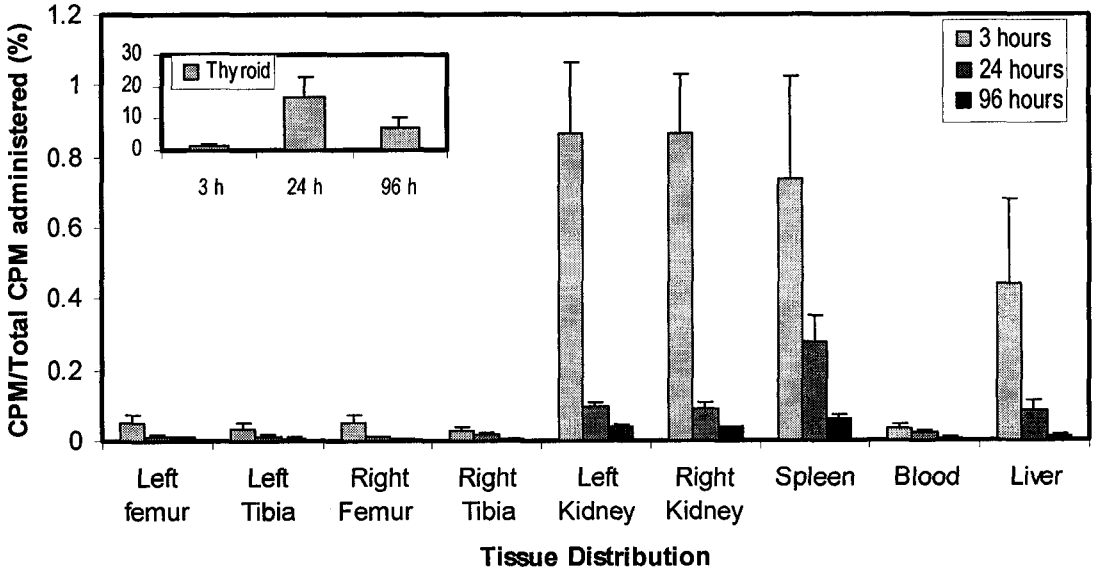


Figure B-2. Pharmacokinetics assessment for BMP-2 after 3, 24 and 96 h after BMP-2 injection.

7.3 APPENDIX C

Objective:

To study the effect of varying the concentrations of the osteogenic supplements β-glycerophosphate (β-GP/ 1 mM, 3 mM, 10 mM) and Dexamethasone (Dex/ 0, 10 nM, 100 nM) on *in vitro* colony calcification of bone marrow stromal cells (BMSC), isolated from 8-month old female Sprague-Dawley rats.

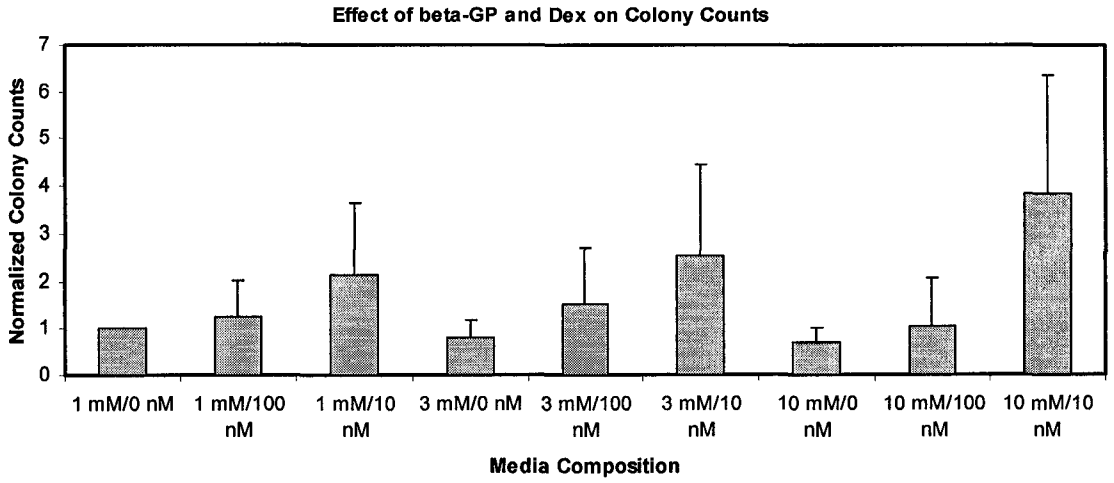


Figure C-1. Effect of varying the concentrations of β-glycerophosphate and Dexamethasone on *in vitro* colony calcification of BMSC.

Objective:

To assess the effect of bFGF an BMP-2 on osteocalcin activity (OCN) in the presence of Dexamethasone in rat bone marrow stromal cells (BMSC).

Bioactivity Model:

1. BMSC isolated from the femurs of 8-month old female rats were seeded in triplicate in 10 cm tissue culture dishes in two different sets of media
   i. 10 mM β-GP and 0 Dex, and ii. 10 mM β-GP and 10 nM Dex
2. Depending on the growth factor treatment group the triplicate set of dishes belonged to they were treated with 10 or 50 ng/mL bFGF OR 150/500 ng/mL BMP-2 for 2 successive weeks. The control group did not receive either growth factor.
3. The cell culture supernatants post 2-week growth factor treatment, which were frozen and stored were used to determine OCN activity using a rat osteocalcin ELISA kit.

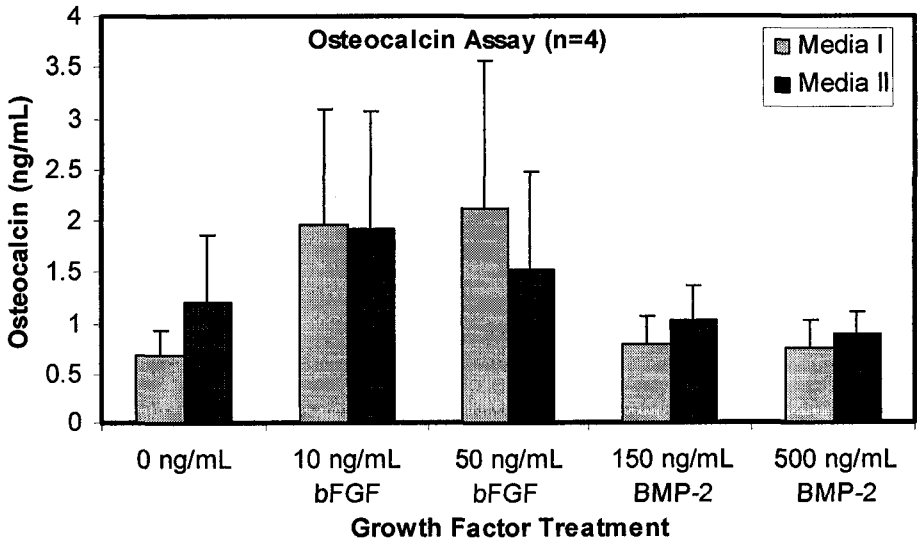


Figure C-2. Effect of bFGF and BMP-2 on osteocalcin activity (OCN) in the presence of Dexamethasone in rat BMSC.

7.4 APPENDIX D

Objective:

To determine the minimum effective dose below 25 μg/kg bFGF that was used in our previous animal study, using BMSC isolated from OVX rats systemically treated with bFGF.

Bioactivity Model:

1. The rats were administered 0, 3 or 10 μg bFGF/injection 5 times a week for 2 weeks. 24 h after the final injection the rats were sacrificed. BMSC were extracted from the left tibia and femur, wherein 1 mL of the 1.5 mL cell suspension was seeded for assays and 0.5 mL was frozen away at -80°C for mRNA extraction.
2. ALP activity and calcium content were assessed at week 2 and 4 of culture, while gene expression for OPN, OCN and BSP were determined at week 1 and 2 of culture and normalized against GAPDH. Gene expression for BSP could not be observed on the agarose gels.

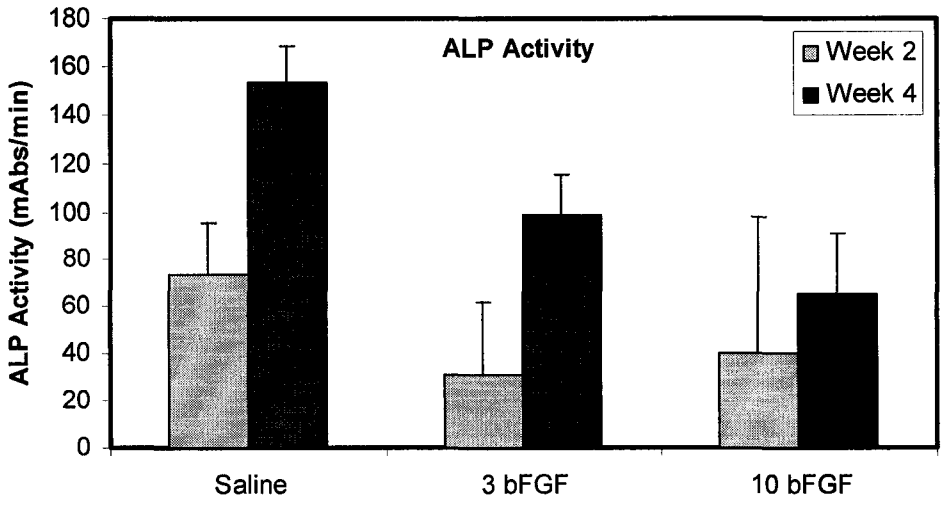


Figure D-1. ALP activity after 2 weeks and 4 weeks of culture of BMSC.

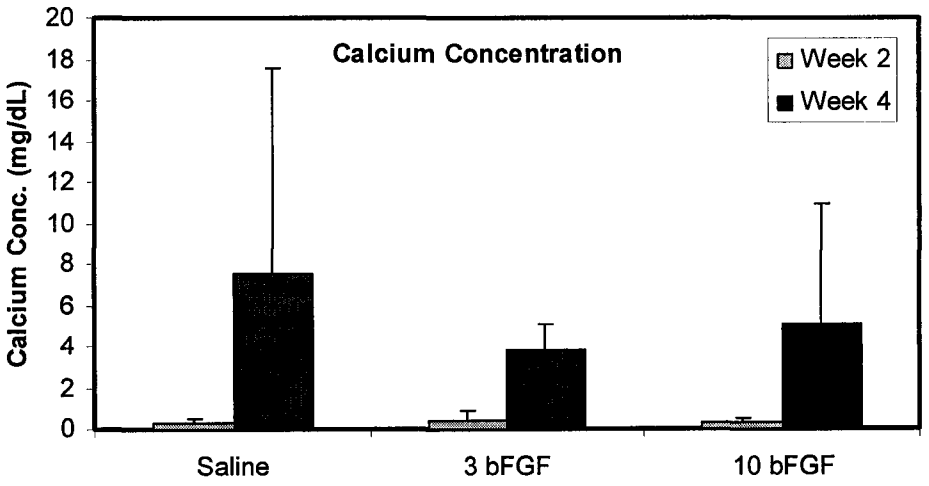
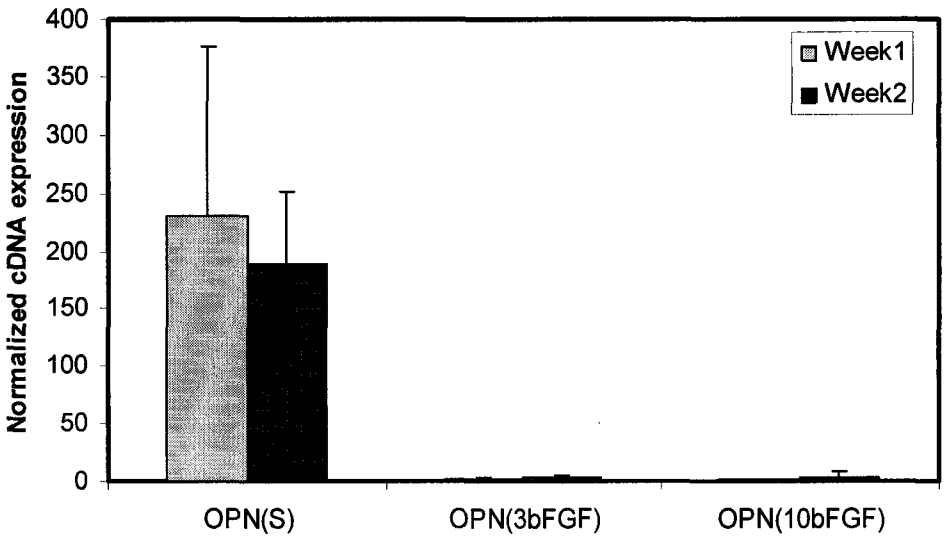


Figure D-2. Calcium content assessed after 2 weeks and 4 weeks of culture of BMSC.

A



B

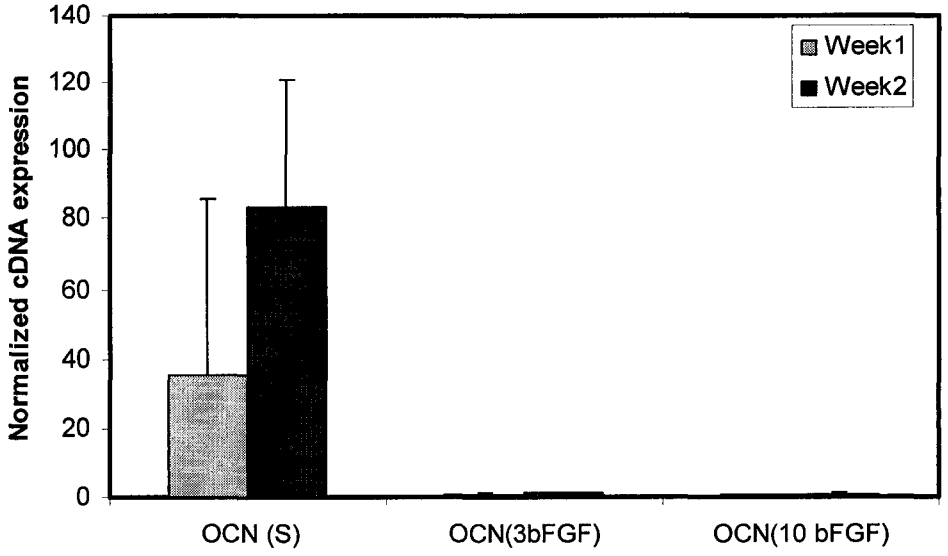


Figure D-3. A. OPN gene expression assessed in the different treatment groups at week 1 and 2 of BMSC culture **B.** OCN gene expression assessed in the different treatment groups at week 1 and 2 of BMSC culture.