# 交通大學生物科技學系

# 博士論文

骨型態發生蛋白的訊息途徑對細胞功能之探討：BMP-4 和流體剪力對

骨母細胞分化作用及腫瘤細胞生長作用之影響

**Controlling Cell Fate by Bone Morphogenetic Protein Signaling:**

**Roles of BMP-4 and Fluid Shear Stress in Osteoblast Differentiation**

**and Tumor Cell Growth**


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# 致　　謝

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骨型態發生蛋白（bone morphogenetic proteins, BMPs）是屬於轉型蛋白（transforming growth factor-beta, TGF-β）中的一種生長因子，因其具有在體內誘導異位（ectopic site）骨頭或軟骨生成作用的能力而被發現與命名，但近幾年研究發現，骨型態發生蛋白在腫瘤細胞上也具有重要的影響性。骨型態發生蛋白-4 為骨格生成作用中最重要的生長因子之一，但經由其他研究發現，骨型態發生蛋白-4 也對其他種類細胞的細胞功能具有調控作用，包含正常細胞和腫瘤細胞的生長或細胞凋亡。機械微環境（mechanical microenvironment）在組織的發展、維持、功能或致病上相當重要，近期研究發現，機械流體力學（mechanical flow forces）對腫瘤細胞的生長具有影響性，也會調控 Smad 訊息途徑來影響細胞功能。此外，科學家在哺乳動物的細胞功能研究上發現，影響細胞週期的運行結果可調控或決定細胞功能的作用。總和以上的論述，我們假設骨型態發生蛋白的訊息途徑可調控細胞週期的運行進而影響骨型態發生蛋白-4 所調節骨母細胞(osteoblasts)增生(proliferation)及分化作用(differentiation)和機械流體力學調節腫瘤細胞生長作用。本論文的研究目的為（1）觀察骨型態發生蛋白-4 是否會調控細胞週期的運行進而調節骨母細胞的分化作用；(2)研究骨型態發生蛋白-4 是否藉由調節細胞週期調控因子（cell cycle regulators）的表現來影響此發生過程；（3）證實細胞膜上的 integrins 是否與骨型態發生蛋白的細胞膜上受體（receptors）交互作用，因而促進及加強骨型態發生蛋白-4 的影響；(4）觀察機械流體力學是否會調控細胞週期的運行進而調節腫瘤細胞的生長作用；(5）研究機械流體力學是否藉由骨型態發生蛋白的訊息途徑來調節細胞週期調控因子的表現進而影響此發生過程；(6）觀察機械流體力學所刺激骨型態發生蛋白的訊息途徑是否是經由刺激細胞自體分泌（autocrine）骨型態發生蛋白所引起；(7）證實細胞膜上科學家認定的機械

接受器（mechanosensors）-integrins 是否為腫瘤細胞接受機械力的受體，因而影響細胞內部骨型態發生蛋白的訊息途徑。

研究發現，骨型態發生蛋白-4 的刺激會遏止兩種不同的骨母細胞的細胞週期，並令其停止在 $G_0/G_1$ 時期，此作用主要是藉由增加細胞週期調控因子 $p21^{CIP1}$ 和 $p27^{KIP1}$ 的蛋白表現所引起，並因此造成細胞進行分化作用。研究中利用小干擾 RNA （small interfering RNA, siRNA）技術發現此作用主要是受到骨型態發生蛋白的訊息途徑：骨型態發生蛋白的膜上第一型受體 A 和 Smad5 所調控。此外，骨型態發生蛋白-4 也會刺激 ERK 基酶的短暫磷酸化，研究發現當 ERK 基酶的活性受到抑制時會抑制骨型態發生蛋白的訊息蛋白 Smad1/5 的活化，且抑制細胞膜上 integrin $\beta_3$ 的表現時，會同時造成骨型態發生蛋白-4 刺激 ERK 和 Smad1/5 的活化被抑制。此結果提出骨型態發生蛋白-4 所造成的反應及訊息途徑會受到 integrin $\beta_3$-ERK 所調控。

在機械流體力學對腫瘤細胞生長調控的研究發現，當給予機械流體力學所產生的剪力刺激四種腫瘤細胞 24 和 48 小時後，會遏止細胞週期的運行，並使其停止在 $G_2/M$ 時期，此作用主要是藉由增加細胞週期調控因子 cyclin B1 和 $p21^{CIP1}$ 的蛋白表現及降低 cyclins A, D1, and E, Cdk-1, -2, -4, and -6, and $p27^{KIP}$ 的蛋白表現以及 Cdk1 的活性所引起。研究中利用抗體（antibodies）和小干擾 RNA 技術發現此作用主要是透過膜上 integrins $\alpha_v\beta_3$ 和 $\beta_1$ 經由骨型態發生蛋白的訊息途徑：骨型態發生蛋白的膜上第一型受體 A 和 Smad1/5 所調控。此外，研究也發現剪力會透過骨型態發生蛋白的訊息途徑抑制腫瘤細胞的分化作用，包含降低轉錄因子 Runx2 與 DNA 結合的活性和分化蛋白骨鈣素（osteocalcin）和鹼性磷酸酶（alkaline phosphatase, ALP）的基因和蛋白表現。

本論文的研究結果證實骨型態發生蛋白的訊息途徑可藉由調控細胞週期的運行而調節骨型態發生蛋白-4 所誘導的骨母細胞分化作用和機械流體力學所誘導的腫瘤細胞

生長抑制作用。也證實機械微環境具有調控腫瘤細胞的分子機制進而影響細胞功能的作用。我們提出對骨型態發生蛋白的訊息途徑活性的調控作用可能對治療骨骼或腫瘤相關疾病提供新的方法，另外，機械流體力學與骨型態發生蛋白的訊息途徑之間的相互關係對於治療腫瘤病人可能也可提供新的研究方向。

## ABSTRACT

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily, were originally identified by their unique ability to induce ectopic bone and cartilage formation *in vivo*. In recent studies, BMP signaling has been suggested to play an important role not only in bone cells but also in tumor cells. BMP-4 is one of the most potent inducers of bone formation and is also demonstrated to be a potent growth factor for modulating other cells' functions, including normal and tumor cells' growth or apoptosis. The importance of the mechanical microenvironment in tissue development, maintenance, function and pathogenesis has been well established for several decades. Recent studies demonstrated that mechanical flow forces may affect the growth of tumor cells. Moreover, it is also suggested that mechanical flow forces may mediate the Smad signaling pathway. In mammals, the regulation of cell cycle distribution can mediate cell functions. We hypothesize that BMP signaling may regulate the cell cycle distribution, thereby mediating osteoblast proliferation and differentiation under BMP-4 treatment and mediating tumor cell growth under mechanical flow forces. The aims of this study were: (1) to observe if BMP-4 regulates the cell cycle distribution in order to induce differentiation in osteoblasts; (2) to investigate if BMP-4 signaling mediates the expression of cell cycle regulators and hence modulates this process; (3) to demonstrate if membrane integrins interact with BMP-4 receptors for optimization of the BMP-4 effects; (4) to observe if mechanical flow forces regulate the cell cycle distribution in order to affect tumor cell growth; (5) to investigate if mechanical flow forces mediate the expression of cell cycle regulators through BMP signaling and hence modulate this process; (6) to observe if mechanical flow force-stimulated BMP signaling comes from BMP autocrine effects; and (7) to demonstrate if the mechanosensor (i.e., integrin) response to mechanical flow forces induces intracellular BMP signaling.

In two osteoblast-like cell lines, BMP-4 stimulation induced $G_0/G_1$ arrest. BMP-4

induced the increased expression of $p21^{CIP1}$ and $p27^{KIP1}$ and hence cell differentiation, but had no effect on the expression of cyclins A, B1, D1, and E, and Cdk-2, -4, and -6. Using specific small interfering RNA (siRNA), we found that the BMP-4-induced $G_0/G_1$ arrest and increased expressions of $p21^{CIP1}$ and $p27^{KIP1}$ were mediated by BMP receptor type IA-specific Smad5. In addition, BMP-4 induced transient phosphorylation of extracellular signal-regulated kinase (ERK); transfection with ERK-specific siRNA inhibited BMP-4-induced Smad1/5 activation. Moreover, transfection with specific siRNA for $\beta_3$, but not $\alpha_v$ and $\beta_1$, integrins inhibited the BMP-4-induced ERK and Smad1/5 phosphorylation, suggesting that the BMP-4-induced responses are mediated by the $\beta_3$ integrin through ERK.

In four tumor cell lines, incubation under static conditions for 24 or 48 h led to $G_0/G_1$ arrest; in contrast, shear stress (12 dynes/cm$^2$) induced $G_2$/M arrest. Shear stress induced increased expression of cyclin B1 and $p21^{CIP1}$ and decreased expression of cyclins A, D1, and E, and cyclin-dependent protein kinases (Cdk)-1, -2, -4, and -6, and $p27^{KIP1}$, as well as a decrease in Cdk1 activity. Using specific antibodies and siRNA, we found that the shear-induced $G_2$/M arrest and corresponding changes in $G_2$/M regulatory protein expression and activity were mediated by $\alpha_v\beta_3$ and $\beta_1$ integrins through bone morphogenetic protein receptor type IA-specific Smad1 and Smad5. Shear stress also down-regulated runt-related transcription factor 2 (Runx2) binding activity and osteocalcin and alkaline phosphatase expression in tumor cells; these responses were mediated by $\alpha_v\beta_3$ and $\beta_1$ integrins through Smad5.

Our findings indicate that BMP signaling may mediate the cell cycle distribution to regulate BMP-4-induced osteoblast differentiation and the mechanical flow forces-induced tumor cell growth arrest. Our findings also provide new insights into the mechanisms by which the mechanical microenvironment modulates molecular signaling, gene expression, the cell cycle, and functions in tumor cells. Modulation of the activities of BMP signaling may be

useful in establishing new approaches to the treatment of a variety of bone or tumor disorders. Moreover, the communications between mechanical flow forces and BMP signaling may contribute new research directions for treating tumor patients, and further detailed investigations are needed.

## BACKGROUND

Cells are constantly influenced by their biochemical and mechanical microenvironments. Chemical ligand binding to their specific receptors under biochemical stimulation and mechanical force regulating membrane mechanosensors, e.g., integrins, can transduce information into the cell and hence modulate cell functions. Thus, modulation in cell signaling, gene expression, structure and function by both chemical and mechanical factors plays important role in health and disease. Bone is living, growing tissue, and is constantly renewed through a two-part process called remodeling. Bone is continuously formed and absorbed by the actions of osteoblasts and osteoclasts, respectively. During bone formation and remodeling, osteoblast and osteoclast function may be regulated by chemical factors, such as BMPs, and mechanical factors, including flow-induced shear stress. Both chemical and mechanical stimuli are essential for the maintenance of skeletal integrity and bone mass. Suppression of these stimuli under conditions such as menopause or spaceflight results in bone loss.

Osteoporosis, osteomalacia, etc, are metabolic bone diseases with major histomorphometric abnormalities: a decreased amount of bone mass and a reduced bone formation rate. Therapy of these diseases is usually inefficiency and requires lone-term treatments. Thus, discovery and establishment of new procedures and methods are urgent. Recent studies suggest that BMPs and flow-induced mechanical forces play important roles in functional modulation of different types of cells, including bone and tumor cells. Studies on the BMP effects demonstrated that BMPs are potential inducers for stimulating osteoblast differentiation and increasing bone mass. BMP-4 has been used clinically to treat the bone fracture healing in patients. Moreover, flow-induced mechanical forces have been proposed to modulate the process of bone formation and remodeling. Interstitial fluid flow in bone, in particular flow-induced shear stress, is required for the maintenance of bone integrity and

serves as a mediator in signal transduction and gene expression during mechanical loading-induced bone remodeling. In physiology, tissues and cells are affected by the interplay between chemical and mechanical stimuli. However, whether combination of BMPs and flow-induced shear stress provides new strategy to treat bone diseases remains to be determined. In this thesis, we investigated the effects of BMPs and flow-induced shear stress on signal transduction and gene expression in bone or tumor cells and the consequent modulation of their functions.

In the present study, we used human MG63 osteoblast-like cells to test our hypothesis. Human MG63 cells have been identified to possess osteoprogenitor potentials of differentiation in response to differentiation signals, tumor cell charactistics with rapid proliferative rate and no aging. In addition to investigate the effects of BMPs and flow-induced shear stress on osteoblast signaling, gene expression and function, we also investigate the effect of shear stress on tumor cell growth. Understanding the detail mechanisms underlying the effects of chemical and mechanical stimuli on bone cell differentiation and tumor cell growth may provide insignts that may be taken into account for therapeutic implication.

# CHAPTER I

# INTRODUCTION

## 1.1 Bone morphogenetic proteins (BMPs)

Bone morphogenetic proteins (BMPs) belong to a large family of structurally related proteins known as the transforming growth factor-β (TGF-β) superfamily, to which TGF-βs, activins, nodal and Mullerian inhibiting substance (MIS)/anti-Mullerian hormone (AMH) also belong [1]. BMPs were originally identified as proteins capable of inducing ectopic cartilage and bone formation when implanted subcutaneously or in muscle pouches [2-3]. This ectopic cartilage/bone formation recapitulates the entire sequence of events that occurs during endochondral bone development in limb buds [4-5]. The activity of BMPs was discovered in 1965, but the BMP proteins were purified and sequenced in the late 1980s [2-3, 6-7]. After that, recombinant BMP proteins were expressed [6, 8]. To date, over 20 BMP family members have been identified and characterized. BMPs were found to have important roles in directing the cell fate choices of mesenchymal cells in vitro. They stimulate osteoblast differentiation and inhibit the differentiation of mesenchymal cells into other cell lineages [9-11]. Subsequent studies showed that BMPs are not only involved in bone formation, but, like other members of the TGF-β superfamily, are multifunctional proteins with effects not related to the formation of bone [12-13]. Correspondingly, BMPs were found to be expressed not only in skeletal tissues, but also in many soft tissues [14]. Consistent with these results, the gain or loss of function studies of BMPs in mice demonstrated that, besides their critical roles in cartilage and bone formation [15-19], BMPs are also important during mouse development and in the adult functions of cardiovascular, pulmonary, reproductive and urogenital organs and in the nervous system [20]. Recent studies suggest that BMPs act as inflammatory cytokines in systemic arteries, promoting endothelial activation, and confirm a striking upregulation of BMPs in atherosclerotic lesions [21-22]. Furthermore, it has been demonstrated that BMPs play important roles during tumor cell growth and metastasis [23]. In light of all these experimental results, it is

suggested that the pleiotropic functions of BMPs implicate a need for the further investigation of their significance in different cell types, including normal and tumor cells.

BMP signaling is mediated by means of the heteromeric complex formation of cognate type I and II transmembrane serine/threonine kinase receptors. Three type I receptors have been shown to bind BMP ligands, including type IA activin receptor (ActRIA or Alk2) and type IA (BMPRIA or Alk3) and IB (BMPRIB or Alk6) BMP receptors [24-26]. Three type II receptors have also been identified, consisting of type II BMP receptor (BMPRII) and type IIA (ActRIIA) and type IIB (ActRIIB) activin receptors [13, 27-28]. Type I and type II BMP receptors are both indispensable for signal transduction. The type II receptors are constitutively active kinases, whereas the type I receptors activate intracellular substrates and thus determine the specificity of the intracellular signals [29-32]. For example, BMP-6 binds exclusively to Alk2, which only activates specific substrates (receptor-regulated Smad proteins, R-Smad, i.e., Smad1 and 5). In contrast, BMP-7 preferentially binds to Alk2, yet has an affinity for Alk3 and 6; thus all three substrates (Smad1, 5, and 8) relay signals. Upon BMP binding, two type receptors form a complex consisting of two pairs of type I and type II receptor complexes [33]. The activation of BMPRII phosphorylates BMPRI, which then leads to the phosphorylation of R-Smads, i.e., Smad1, 5, and 8. The activated R-Smads interact with the common partner Smad4 (Co-Smad) and accumulate in the nucleus to regulate the transcription of different target genes depending on the transcriptional co-modulators recruited [29-35]. R-Smads can directly bind to DNA, however, the affinity is relatively low and an interaction with sequence-specific DNA binding proteins is critical for the formation of a stable DNA-binding complex [36]. The first demonstration that Smads can directly bind to DNA was reported in *drosophila* [37]. After that, it was also suggested that R-Smads may interact with bone-specific transcription factor Runx2 and activate the transcription of target genes such as alkaline phosphatase (ALP), osteocalcin (OCN), or

COX-2 and type X collagen (Col-X) in osteoblasts or in chondrocytes [38-42]. Like other signal transduction pathways, BMP signaling is subjected to modulation at multiple levels. Extracellularly, secreted BMP antagonists, such as noggin, chordin, follistatin, and sclerostin, control the local concentration of active BMPs by sequestration [34, 43]. Within the cytoplasm, inhibitory Smads (I-Smads, i.e., Smad 6 and 7) interfere with R-Smad and Co-Smad complexes [43], and Smurfs (Smad ubiquitination regulatory factors) target activated receptors and R-Smads for degradation [43-44].

Although abundant evidence demonstrates that Smads are critical for BMP signaling, accumulating data suggests that Smad-independent pathways may also exist. Recently, an alternative BMP-mitogen-activated protein kinase (MAPK) signaling pathway [45-47], including extracellular signal-regulated kinase (ERK), has been described. In vitro and cell culture studies have revealed that ERK may phosphorylate serine residues of Smad1 [48]. As a consequence of this phosphorylation, the nuclear accumulation of Smad1 is inhibited in cultured cells, although the mechanism for this inhibition is unclear. Thus, it appears that ERK can crosstalk with BMP signaling by differentially phosphorylating Smad1 to affect its nuclear localization. In addition, recent studies in osteoblasts also reported that ERK may mediate the signaling pathway of BMP-4 in regulating the synthesis of fibronectin and extracellular fibronectin fibrillogenesis, which is involved in the process of bone mineralization [49]. The importance of ERK in BMP signaling effects has already been demonstrated, however, the detailed mechanisms and the correlation between Smad and ERK pathways in response to BMPs remain unclear. Membrane integrins are the other BMP signaling mediators. Integrins, as the main receptors that connect the cytoskeleton and the extracellular matrix (ECM), have been shown to play important roles in modulating gene expression and cellular functions in a wide variety of cells seeded on the ECM [50]. Osteoblasts express several types of integrins, including $\alpha_v\beta_3$ and those containing the $\beta_1$

subunit (dimerized with α subunits, including $\alpha_1$, $\alpha_3$, $\alpha_5$, and $\alpha_6$) [51-53], which have been shown to play important roles in osteoblast commitment and differentiation [54]. The administration of the RGD (Arg-Gly-Asp) peptide, which is a non-specific inhibitor of integrins, has been shown to inhibit bone formation and resorption of fetal rat parietal bones [55]. The interaction between integrins and fibronectin has been shown to be required for calvarial osteoblast differentiation [56-57]. Recent studies using function-perturbing antibodies against integrins showed that $\alpha_v\beta$ and $\alpha\beta_1$ integrins play an essential role in BMP-2-induction of osteoblast differentiation [54]. It is likely that integrins cooperate with BMP receptors to mediate the BMP-eliciting signaling pathways in osteoblasts and hence modulate their gene expression and cellular functions.

### 1.2 BMP signaling in osteogenesis

During Osteogenesis, bone is formed in two different manners: intramembranous ossification and endochondral ossification [30, 58]. In the case of intramembranous ossification, osteogenesis occurs directly through the differentiation of mesenchymal stem cells to mature osteoblasts. Ossification generated in this fashion is responsible for forming the flat bones of the skull, part of the clavical, and the additional bone on the periosteal surface of long bones. In the process of endochondral ossification, mesenchymal stem cells first condense to form a cartilage model, and then bone formation occurs by replacing this cartilage. This type of ossification forms most of the bones, including the axial and appendicular skeletons. A fundamental function of BMP signaling is to induce the differentiation of mesenchymal stem cells toward cells of the osteoblast lineage to promote osteoblast maturation and function. To determine the importance of BMP ligands, receptors and signaling proteins in embryonic development and bone formation, null mutations affecting BMP signaling have been created and the phenotypic changes in the animals have been extensively studied. BMP-4 knockout mice die during early gastrulation due to failure

of mesodermal differentiation and pattern defects [59]. BMP-2/-4 conditional knockout (CKO) mice show severe defects in bone development [60]. Similarly BMP-4 CKO mice also show defects in bone development and postnatal bone formation [61]. Transgenic mice that overexpress noggin, BMPs' antagonist, in osteoblasts have reduced bone mineral densities and bone formation rates [62-63]. Transgenic mice expressing truncated dominant-negative BMP receptors have been shown to develop postnatal osteopenia [64]. Deleting the Smad1 gene specifically in osteoblasts causes a reduction in bone mass [65] and mice with disruption of Smad5 develop multiple embryonic defects [66].

Osteogenesis and bone remodeling are complex process that begin in the embryo and continue in the adult to maintain the balance between bone formation and resorption. These processes include osteoblast proliferation, differentiation, and apoptosis [67-68]. Evidence is accumulating that shows mammalian progenitor cell proliferation depends on the cell cycle progression and that they must exit the cell cycle to differentiate into mature cells or program to death [69]. Therefore, these cell functions may be closely linked and all regulated by cell cycle distribution. BMPs have the unique function of inducing the differentiation of the osteoblast lineage, leading to an increase in the pool of mature cells. Recently, conventional gene knockout experiments have shown that BMPs have diverse biological activities in a variety of cell types, mediated by their ability to regulate cell proliferation and apoptosis [70-71]. Therefore, scientists suggest that the effects of BMPs on osteogenesis may be global, including the modulation of osteoblast proliferation, differentiation, and apoptosis, and that these effects and correlations need to be further clarified.

The proliferation of eukaryotic cells depends on their progression through the cell cycle, and cell cycle arrest at the $G_0/G_1$ phase is thought to be a prerequisite for cell differentiation and apoptosis [69]. Cell cycle is controlled by regulatory proteins, including cyclin-dependent protein kinases (Cdks) and their regulatory subunits, cyclins, as well as

inhibitors such as $p21^{CIP1}$ and $p27^{KIP1}$ [72]. Recent studies demonstrated that $p21^{CIP1}$ and $p27^{KIP1}$ inhibit the activities of all Cdks and regulate cell proliferation and differentiation [73]. Halevy et al. [74] demonstrated that MyoD, a skeletal muscle-specific transcription regulator, induces cell cycle arrest during skeletal muscle differentiation by increasing the expression of $p21^{CIP1}$. Using osteoprogenitor cells derived from the bone marrow of $p27^{-/-}$ mice, Drissi et al. [75] demonstrated that $p27^{KIP1}$ plays a key role in the regulation of osteoblast differentiation by controlling proliferation-related events. Although $p21^{CIP1}$ and $p27^{KIP1}$ have been shown to play roles in regulating cell proliferation and differentiation, the functional significance of $p21^{CIP1}$ and $p27^{KIP1}$ in modulating the response of osteoblasts to BMP signaling have not been reported. Moreover, the mechanisms that regulate the expression of cell cycle regulatory proteins in osetoblasts in response to BMP signaling have not been fully clarified.

### 1.3 BMP signaling in tumor biology

In addition to normal cell regulation, some studies have suggested a role for BMP signaling in tumor growth and metastasis. The most striking indication that BMP signaling contributes to tumorigenesis comes from genetic studies of familial tumor syndromes. Mutations of Smad4 and BMPRIA are genetically responsible for familial juvenile polyposis [76-77]. Germ line mutations in BMPRIA have been identified in a subset of families with Cowden syndrome, an inherited breast tumor syndrome [78]. Recent studies even demonstrate that BMP signaling may inhibit the tumorigenic potential of tumor stem cells, including brain and glioma [79-80]. In addition, in prostate tumor tissues, increased levels of expression of BMP-6 and -7 have been correlated with bone metastasis [81-82], while another laboratory reported an inverse correlation between the abnormal expression of BMPRs and the pathological grade of the prostate tumor [83]. The loss of expression of BMPRII is associated with a higher tumor grade and pathological stage, an increased rate of

recurrence, and a decreased rate of survival after surgery [84]. In breast tumor tissues, the expression of BMPRIB has been proposed to be a major marker of progression and de-differentiation [85], while the overexpression of BMP-4 has been shown in colorectal adenomas [86]. Likewise, the coexpression of BMP-2/-4 and BMPRII has been reported to be associated with a poor prognosis in osteosarcoma patients [87]. It has been shown that various sporadic human tumors also exhibit aberrations in BMP signaling (summarized in Table 1-1) [88].

In tissue culture, the effect of BMP signaling varies between cell types and culture conditions. For example, among prostate tumor cell lines, BMP-2 inhibited the proliferation of the androgen-sensitive LNCaP but not the androgen-insensitive PC3 and DU145 cells [89]. Similarly, BMP-4 treatment decreased the rate of proliferation of LNCaP but not PC3 cells [90]. In contrast, BMP-6 inhibited the proliferation of both androgen-sensitive and –insensitive cell lines [84]. More recently, BMP signaling has been shown to enhance bone invasion by prostate tumor cells [91]. Despite the tremendous progress achieved in delineating the functional significance of BMP signaling in tumorigenesis during the last decade, the mechanism of BMP signaling in tumorigenesis is still not very clear. Compelling evidence indicates that the regulation of BMP signaling in tumorigenesis is cell-specific, and both pro-tumor and anti-tumor effects have been described. Thus, the precise effects and mechanisms of BMP signaling in malignant cells must be further demonstrated, and must be interpreted in the context of cell types and experimental conditions.

### 1.4 Mechanical flow force biology

The importance of microenvironmental dynamic mechanical stresses in tissue development, maintenance, function and pathogenesis has been well established for several decades [92-93]. Fluid flows and pressure gradients that are present in all living tissues

drive blood, lymph, or interstitial flow through blood vessels, lymphatic tube, or extracellular matrix (ECM), respectively [94]. These fluid flows not only help to transport nutrients, drugs, and wastes, but also have important roles in tissue homeostasis and cell biology, including embryonic development, tissue morphogenesis and remodeling, inflammation, lymphedema, tumor biology and immune cell trafficking [94-95]. The mechanisms of mechanical flow forces are extensive studied in vascular cells. Endothelial cells (ECs) are constantly subjected to blood flow-induced shear stress [96-99]. The nature and magnitude of the shear stress plays a significant role in the homeostasis of the structure and function of the blood vessel. Laminar blood flow with a high shear stress modulates cellular signaling and EC functions, and is protective against inflammatory reactions. In contrast, oscillatory blood flow and low shear stress stimulate the expression of EC genes that promote inflammation. This suggests that mechanical flow forces may modulate the intracellular signaling and gene expressions that regulate the ECs' activities [96-99]. During this process, integrins may be the most likely membrane mechanosensors [100]. Integrins have been shown to play an important role in transmitting mechanical flow forces into chemical signals in a variety of cells seeded on an ECM [100-101]. In several systems, including ECs, shear-activation of integrins leads to an increase in their association with Shc and focal adhesion kinase (FAK), which subsequently activate several intracellular signaling cascades, including ERK signaling [100]. In addition, there is evidence that integrins may be cooperative with the receptors of several growth factors, including insulin receptor and platelet-derived growth factor-β receptor, to form integrin-receptor heteromeric complexes that mediate downstream signaling cascades under shearing [102]. However, the detailed mechanisms of the association between integrins and growth factor receptors under flow are not clear. Scientists also suggest that mechanical flow forces may modulate the association between integrins and membrane receptors, and hence modulate the downstream signaling, in

all cell types, including tumor cells. Recent studies demonstrated that mechanical flow forces may stimulate the Smad pathway to mediate EC differentiation [103-104]. Brown JD et al. demonstrated that shear stress induced Smad2 activation through the MAPK pathway in ECs [103]. However, Wang H et al. reported that shear stress down-regulated Smad2, 3, and 4, and up-regulated Smad7 activities, to induce the differentiation of mesenchymal stem cells into ECs [104]. The detailed mechanisms and correlations between mechanical flow forces and BMP signaling in endothelial cells, or even others cells, is still unclear.

### 1.5 Mechanical flow forces and tumors

Solid tumors consist of a porous interstitium and a neoplastic vasculature composed of a network of capillaries with highly permeable walls [105-107]. Fluid flows across the vasculature and enters the tumor by convective and diffusive extravasation through the permeable capillary walls. Tumor fluid flows are characterized by spatial and temporal heterogeneities, and can significantly affect tumor growth, metastasis, and therapy [105]. The characteristics of tumor fluid flows are such that (1) fluid flows are diverted away from the center of the tumor toward a more peripheral path, and (2) at a fixed location, the fluid flows are not uniform with time, showing an intermittent flow pattern and flow rate and even periodic inversions of the direction of flow. Despite the important role of tumor fluid flows in tumor physiopathology and treatment, to date there are no complete explanations for the observed tumor fluid flow anomalies.

Although millions of cells are shed from a tumor every day, metastasis is believed to be very inefficient [108]. This inefficiency is widely assumed to be a result of the destruction of cells by both fluid flow and the immune system. The circulation itself represents a highly toxic environment for disseminating tumor cells. The mechanical destruction of circulating tumor cells is the first line of defense in the host microenvironment acting against tumor spreading. Tumor cells circulating in the blood are subjected to intense mechanical stresses

by the shear stress caused by blood flow. Especially in narrow capillaries, the required sphere-to-cylinder shape-transformation is lethal to a majority of tumor cells. These shear forces acting on tumor cells are very intense in small capillaries. At the same time, this deformation of circulating cells within the narrow capillaries enables an intense contact of the tumor cell's surface adhesion molecules with potential ligands at the capillary walls, and tumor cell adhesion may be initiated. This is caused by the enlarged contacting surface areas of deformed cells, resulting in the increased availability of cell adhesion molecules, and a longer time for the establishment of adhesive bonds. Therefore, the balance between the mechanical destruction and the adhesion initiation by mechanical forces on tumor cells appears to be a regulatory mechanism of tumor spreading [108].

Despite the influence of mechanical flow forces on tumor pathobiology and drug delivery having been studied, the effect of the flow-induced shear force on tumor cells has not been explored much. Compressive forces have been shown to inhibit tumor cell growth [109] and up-regulate adhesion molecules [110]. A recent study reported that tumor cell proliferation is affected by intratumoral pressure, and that the activations of mitogen-activated protein kinases and nuclear antigen Ki-67 are involved in this mechanical modulation [111]. While these results show that mechanical forces can modulate tumor cell responses, the detailed mechanisms by which mechanical stimuli are transduced into cellular signaling to regulate the tumor cell gene expression and functions remain unclear.

**Table 1-1 Aberrations of BMP signaling in human tumor, the functional relevance and clinical correlations.** (From Hsu MY. et. al., *Cancer Metastasis Rev.* 2005;24: 251-263.)

| Tumor type | Aberrations | Functional implications | Clinical outcome |
|---|---|---|---|
| Colorectal | BMP2↓ | BMP2 serves as a tumor suppressor | |
| | Smad4↓ | | advanced disease, ↑lymph node metastasis, and ↓ survival |
| | Smad7↑ | | ↓survival |
| | Smad8↓ | | |
| Breast | BMP2↓ | BMP2 serves as a tumor suppressor | |
| | BMP7↑ | | Tumor differentiation |
| | Smad8↓ | | |
| Non-small cell lung | BMP2↑ | ↑migration, invasion, and growth | |
| Pancreatic | BMP2↑ | BMP2 is mitogenic | |
| | Smad4 mutation | | |
| | Alk3 and BMPRII↑ | | |
| Gastric | BMP4↑ | | Tumor dedifferentiation |
| Oral papilloma /Squamous cell carcinoma | BMP2, 4, and 5, and Alk3↑ | | High risk for aggressive disease |
| Esophageal squamous cell carcinoma | BMP6↑ | | Tumor dedifferentiation and poor prognosis |
| | Smad4↓ | | ↑tumor depth and lymph node metastasis |
| | Smurf2↑ | | Poor prognosis |
| Prostate | BMP6↑ | | ↑recurrence; ↓survival |
| | BMP7↑ | | Bone metastasis |
| | BMPR↓ | | Tumor dedifferentiation |
| Nephroblastoma | BMP7↓ | | |
| Malignant glioma | BMP7↑ and Alk3↑ | BMP7 is growth-inhibitory | Tumor dedifferntiation |
| Cervical | Smad4↓ | | |
| Hepatocellular | Smad4↓ | | |
| | Glypican3↑ | | |
| Endometrial | Smad4↓ | | |
| Leukemia | Smad5↓ | | |
| Thyroid follicular | Smad7↑ | | |
| Melanoma | Ski↑ | Ski acts as an oncogene | |
| Renal cell carcinoma | BMPRII↓ | resistance to growth inhibition by BMP6 | |

# CHAPTER II

# HYPOTHESIS AND SPECIFIC AIMS

BMP signaling is a multifunctional mechanism. It is important for bone formation, including osteoblast maturation (i.e., differentiation and proliferation) and tumor cell growth and metastasis inhibition. The BMP-4 synthesized by osteoblasts is one of the most potent inducers of bone formation through its stimulation of osteoblast differentiation. BMP-4 was also demonstrated as a potential growth factor for modulating other cell types' functions, including normal and tumor cells' growth or apoptosis. Mechanical flow forces were originally extensively studied in blood biology. Recent studies demonstrate that mechanical flow forces also affect the biophysics and physiology of other cells or tissues, including bone cells and tumor cells. Moreover, it is also suggested that mechanical flow forces may mediate the Smad signaling pathway. In mammals, the regulation of the cell cycle distribution can mediate cell functions; cell proliferation depends on the cell cycle progression, but the cell cycle must be arrested prior to cell differentiation and apoptosis. In light of all this information, we propose that BMP signaling may regulate the cell cycle distribution to mediate osteoblast proliferation and differentiation under BMP-4 treatment, and to mediate tumor cell growth under mechanical flow force stimulation. To test our hypothesis, these seven specific aims were proposed.

1. To observe if BMP-4 regulates the cell cycle distribution in order to induce differentiation in osteoblasts.
2. To investigate if BMP-4 signaling mediates the expression of cell cycle regulators and hence modulates this process.
3. To demonstrate if membrane integrins interact with BMP-4 receptors to optimize the BMP-4 effects.
4. To observe if mechanical flow forces regulate the cell cycle distribution in order to affect tumor cell growth.
5. To investigate if mechanical flow forces mediate the expression of cell cycle

regulators through BMP signaling and hence modulate this process.

6. To observe if the mechanical flow force-stimulated BMP signaling comes from BMP autocrine effects.

7. To demonstrate if the mechanosensor, i.e., integrin, response to mechanical flow forces induces the intracellular BMP signaling.

# CHAPTER III

# MATERIALS AND METHODS

### 3.1 Cell lines and cell culture

Human MG63 osteoblast-like cells were osteosarcoma cells that were isolated in 1977 [112]. The cell line has been identified to possess osteoprogenitor potential for differentiation in response to differentiation signals, tumor cell character with rapid proliferation, and no aging [112-113]. Therefore, the MG63 cells were cultured to study the BMP-4 and mechanical flow force effects in the present thesis. Mouse MC3T3-E1 osteoblast-like cells were a positive control for the BMP-4 treatment experiments. Saos2 osteosarcoma cells, SCC25 oral carcinoma cells, and SW1353 chondrosarcoma cells were positive controls for the mechanical flow force experiments. All cell lines were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco). In the BMP-4 treatment experiments, cells (~1-2×$10^5$ cells/$cm^2$) were trypsinized and seeded onto the 60 mm culture dish. The medium was exchanged with a medium that was identical except that it contained only 0.5% FBS, and the cells were further incubated for 24 h before treatment with BMP-4. In the mechanical flow force experiments, cells (~1-2×$10^5$ cells/$cm^2$) were trypsinized and seeded onto glass slides (75x38 mm; Corning, NY) pre-coated with type I collagen (30 μg/ml). The medium was then exchanged with DMEM containing only 2% FBS for the 24 h incubation of the cells prior to the experiment.

### 3.2 Materials

Mouse monoclonal antibodies (mAbs) against cyclin E (sc-25303), Cdk2 (sc-748), ERK2 (sc-1647), phospho-ERK (sc-7383), and $\beta_3$ integrin (sc-46655), and goat polyclonal antibodies (pAbs) against Smad1/5 (sc-6031), Runx2 (sc-12488) and $\alpha_v$ integrin (sc-10719), were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAbs against

cyclins A (#4656), B1 (#4135), and D1 (#2926), Cdk-4 (#2906) and -6 (#3136), and $p21^{CIP1}$ (#2946), and rabbit pAbs against $p27^{KIP1}$ (#2552), Cdk1 (#9112), phospho-Cdk1 (Y15) (#9111), and phospho-Smad1/5 (#9511), were purchased from Cell Signaling Technology (Beverly, MA). Mouse mAbs against $\alpha_v\beta_3$ (MAB1976) and $\beta_1$ (MAB2253) integrins were purchased from Chemicon (Temecula, CA). The dominant-negative mutants of Shc (Shc-SH2) and FAK [FAK (F397Y)] were previously described [114-115]. The OCN promoter construct was a gift from Dr. Leland WK Chung (Department of Urology, Emory University School of Medicine). The control siRNA and specific siRNAs of BMPRIA, BMPRIB, Smad1, Smad5, $p21^{CIP1}$, $p27^{KIP1}$, ERK, and $\alpha_v$, $\beta_1$, and $\beta_3$ integrins were purchased from Invitrogen (Carlsbad, CA). Recombinant human BMP-4 was purchased from R & D Systems (Minneapolis, MN). All other chemicals of reagent grade were obtained from Sigma (St Louis, MO), unless otherwise noted.

### 3.3 Flow apparatus.

The glass slide with cultured tumor cells was mounted in a parallel-plate flow chamber characterized and described in detail elsewhere (Figure 3-1) [116]. The chamber was connected to a perfusion loop system, kept in a constant-temperature controlled enclosure, and maintained at pH 7.4 by continuous gassing with a humidified mixture of 5% $CO_2$ in air. The fluid shear stress ($\tau$) generated on the cells by the flow was estimated to be 12 dynes/$cm^2$, unless otherwise noted, using the formula $\tau = 6\mu Q/wh^2$, where $\mu$ is the dynamic viscosity of the perfusate, Q is the flow rate, and h and w are the channel height and width, respectively.

### 3.4 Flow cytometric analysis.

The cells were harvested in PBS containing 2 mM ethylenediaminetetraacetic acid, washed once with PBS, and fixed for 30 min in cold ethanol (70%). Fixed cells were washed and permeabilized with 0.1% Triton X-100 in PBS. They were then stained with 50

μg/ml of propidium iodide (Roche, Basel, Switzerland) and 1 mg/ml RNase A for 30 min. Stained cells were analyzed with a fluorescence-activated cell sorter (FACS) Calibur (Becton-Dickinson, Franklin Lakes, NJ), and the data were analyzed using a mod-fit cell cycle analysis program.

### 3.5 ALP specific activity assay.

The cell extract was prepared with 0.1% Triton X-100 after the shear stress experiments. Cellular ALP activity was assayed at the end of the incubation with 10 mM *p*-nitrophenyl phosphate in 0.15 M sodium carbonate buffer (pH 10.3) and 1 mM $MgCl_2$, as previously described [117], and was normalized against the cellular protein determined by the Bio-Rad protein assay.

### 3.6 RNA isolation and quantitative real-time PCR.

The total RNA was isolated by the guanidium isothiocyanate/phenochloroform method and converted to cDNA as described [97]. The cDNA was amplified through PCR on a LightCycler (Roche Diagnostics, East Sussex, United Kingdom) using LightCycler FastStart DNA MasterPlus SYBR Green I (Roche Diagnostics) with 0.5 μM primers of OCN (sense: 5′-TGAGAGCCCTCACACTCCTC-3′; antisense: 5′-ACCTTTGCTGGACTCTGCAC-3′; product length, 98 bp), ALP (sense: 5′- CAACCCTGGGGAGGAGAC-3′; antisense: 5′-GCATTGGTGTTGTACGTCTTG-3′; product length, 78 bp), and β-actin (sense: 5′-AAATCGTCCGTGACATCAAG-3′; antisense: 5′- GGAAGGAAGGCTGGAAGAGA -3′; product length, 180 bp) genes. PCR was performed in triplicate at 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 5 sec, extension at 72°C for 8 sec, and single signal acquisition for 10 sec. β-actin gene expression was used as an internal control. The PCR conditions were optimized to obtain a PCR product with a single peak on the melting curve analysis on the LightCycler. The raw data collected from

the LightCycler were analyzed using LightCycler Software Version 3.5 (Roche Diagnostics). The gene expression levels were normalized with the β-actin gene expression levels in the same sample.

### 3.7 Western blot analysis.

The cells were collected by scraping and lysed with a buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor mixture (PMSF, aprotinin, and sodium orthovanadate). The total cell lysate (100 μg of protein) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-μm pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed using the Western-Light chemiluminescent detection system (Applied Biosystems, Foster City, CA).

### 3.8 Immunoprecipitation.

The cells were scraped and lysed with a buffer containing 25 mM HEPES, pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.125 M NaCl, 5 mM EDTA, 50 mM NaF, 1 mM $Na_3VO_4$, 1 mM PMSF, 10 mg/mL leupeptin, and 2 mM BGP. The cells were disrupted on ice by repeated aspiration through a 21-gauge needle. The same amount of protein from each sample was incubated with a designated antibody for 2 h at 4°C with gentle shaking. The immune complex was then incubated with protein A/G plus agarose for 1 h and collected by centrifugation. These agarose-bound immunoprecipitates were washed and incubated with boiling sample buffer containing 62 mM Tris-HCl, pH 6.7, 1.25% (w/v) SDS, 10% (v/v) glycerol, 3.75% (v/v) mercaptoethanol, and 0.05% (w/v) bromphenol blue. The samples were then subjected to SDS-PAGE and Western blotting.

### 3.9 Treatments with RGD peptides and mAbs.

Type I collagen contains the integrin-recognition tripeptide RGD (Arg-Gly-Asp) sequence [118]. To block specific integrin-collagen interactions, the cells were pre-incubated with the tetrapeptide RGDS (Arg-Gly-Asp-Ser; 500 μg/mL), which blocks cell adhesion through the RGD sequence on ECM proteins, or the antibodies (10 μg/mL) against $\alpha_v\beta_3$ and $\beta_1$ integrins for 2 h before seeding onto the glass slides pre-coated with type I collagen and during the application of fluid flow.

### 3.10 Reporter gene construct, DNA plasmids, siRNA, transfection, and luciferase assay.

The OCN promoter construct (OCN-Luc) contains 800 bp of OCN 5′-flanking DNA linked to the firefly luciferase reporter gene of the plasmid pGL3 (Promega Inc.) [119]. This fragment of the OCN promoter contains Runx2 binding sites. DNA plasmids at a concentration of 1 μg/mL were transfected into MG63 cells at 60% confluence by using lipofectamine (Gibco). The pSV-β-galactosidase plasmid was co-transfected to normalize the transfection efficiency. The cells were kept as static controls or subjected to shear stress experiments 48 h after transfection. For siRNA transfection, MG63 cells at 70-80% confluence were transfected with the designated siRNA at various concentrations (5, 15, 30, and 40 nM) using the RNAiMAX transfection kit (Invitrogen, Carlsbad, CA).

### 3.11 Electrophoretic mobility shift assay (EMSA).

The cells were collected by scraping in PBS. After centrifugation of the cell suspension at 2000 rpm, the cell pellets were resuspended in cold buffer A (containing, in mmol/L, KCl 10, ethylenediamine tetraacetate [EDTA] 0.1, dithiothreitol [DTT] 1, and phenyl methylsulfonyl fluoride [PMSF] 1) for 15 min. The cells were lysed by adding 10% NP-40 and then centrifuged at 6000 rpm to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer B (containing, in mmol/L, 4-(2-hydroxyethyl)-1-piperazine-

ethane-sulfonic acid [HEPES] 20, EDTA 1, DTT 1, PMSF 1, and NaCl 400), vigorously agitated, and then centrifuged. The supernatant containing the nuclear proteins was used for the EMSA or stored at -70°C until used. A sequence of 20 bp-oligonucleotides containing the human OCN Runx2 site was synthesized (5′-CGTATTAACCACAATACTCG-3′ and 5'-AATTGGTGTTATGAGCATGC-3') [120]. The oligonucleotides were end-labeled with [γ-$^{32}$P]ATP. The extracted nuclear proteins (10 μg) were incubated with 0.1 ng $^{32}$P-labeled DNA for 15 min at room temperature in 25 μL binding buffer containing 1 μg poly(dI-dC). In the antibody supershift assay, an antibody against Runx2 (1 μg each; Cell Signaling Technology) was incubated with the mixture for 10 min at room temperature, followed by the addition of the labeled probe. The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels. The gels were dried and imaged by autoradiography.

### 3.12 Statistical analysis.

The results are given as the mean±SEM. Statistical tests were performed with an independent Student t-test for two groups of data and the analysis of variance (ANOVA) followed by Scheffe's test for multiple comparisons. A $P$ value < 0.05 was considered significant.

**Figure 3-1**

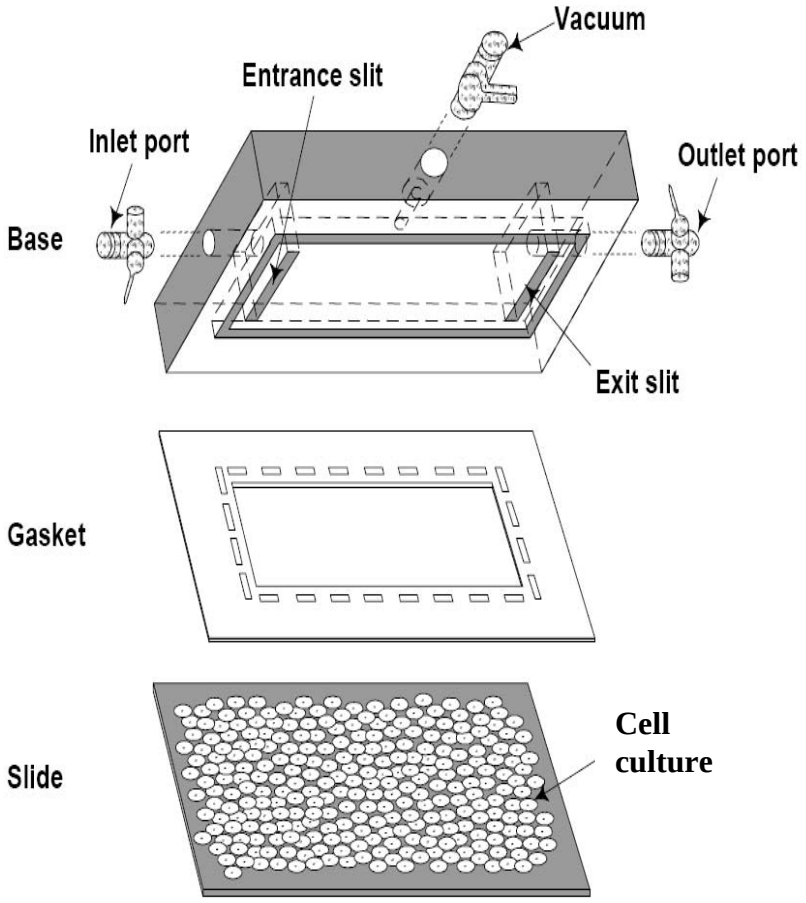



**Figure 3-1 Schematic diagram of the cell culture flow model.** Tumor cells were seeded on the type I collagen gel on the glass slide.

# CHAPTER IV

# RESULTS

## 4.1 Bone Morphogenetic Protein-4 Induces Osteoblast $G_0/G_1$ Arrest and Differentiation via Increased Expression of $p21^{CIP1}$ and $p27^{KIP1}$: Roles of integrins, extracellular signal-regulated kinase, and Smad

***4.1.1 BMP-4 induces $G_0/G_1$ arrest and increased expression of $p21^{CIP1}$ and $p27^{KIP1}$ in osteoblast-like cells.*** To determine the regulatory effect of BMP-4 on the cell cycle in osteoblasts, human MG63 and mouse MC3T3-E1 osteoblast-like cells were kept as controls or treated with BMP-4 (25 ng/ml) for 24, 48, 72, and 96 h, and their cell cycle distributions were analyzed using flow cytometry. The incubation of these cells under static conditions for 48, 72, or 96 h led to an increase in the cell percentage in $G_0/G_1$ phases and a decrease in synthetic and/or $G_2$/M phases (Table 4-1). The treatment of these cells with BMP-4 caused a significant increase in the cell percentage in the $G_0/G_1$ phases and decreases in the synthetic and/or $G_2$/M phases compared with the untreated control cells for the same periods. These results suggest that BMP-4 induces $G_0/G_1$ arrest in these osteoblast-like cells. The changes of the cell cycle distribution in MG63 cells induced by BMP-4 were consistent with the concentration range of the BMP-4 used (i.e., 10, 25, 50, and 100 ng/ml), indicating that the BMP-4-induced $G_0/G_1$ arrest was dose-independent over the range tested (Table 4-2).

We investigated the molecular basis of this BMP-4 effect, and the presentation is focused on the human MG63 cells. The treatment of the MG63 cells with BMP-4 (25 ng/ml) for 24, 48, 72, or 96 h resulted in increases in $p21^{CIP1}$ and $p27^{KIP1}$ expression in these cells (Figure 4-1). In contrast, BMP-4 did not have effects on the expression of cyclins A, B1, D1, and E, and Cdk-2, -4, and -6 in the MG63 cells.

***4.1.2 BMP-4-induced MG63 cell differentiation is mediated by $p21^{CIP1}$ and $p27^{KIP1}$.*** Since cell cycle regulator-led inhibition of the $G_0/G_1$-to-synthetic phase transition has been shown to be critical for cell differentiation [73], and since we have demonstrated that BMP-4 induces

$G_0/G_1$ arrest in MG63 cells with concomitant increases in $p21^{CIP1}$ and $p27^{KIP1}$ expression, we investigated whether the BMP-4-induced MG63 cell differentiation is mediated by $p21^{CIP1}$ and $p27^{KIP1}$. MG63 cells were kept as controls or treated with BMP-4 (25 ng/ml) for 24, 48, 72, and 96 h, and their expression or activity levels for differentiation markers, i.e., osteocalcin (OCN) and alkaline phosphatase (ALP), were examined. Treatment with BMP-4 for 24, 48, 72, and 96 h resulted in significant increases in OCN gene expression and ALP activity in MG63 cells (Figure 4-2A). These BMP-4-induced increases in OCN expression and ALP activity were abolished by the transfection of cells with $p21^{CIP1}$- and $p27^{KIP1}$-specific siRNAs (40 nM for each) (Figure 4-2B), which had 80-90% blocking effects on their respective protein expressions (Figure 4-2C). These results suggest that the BMP-4-induced MG63 cell differentiation is mediated by $p21^{CIP1}$ and $p27^{KIP1}$.

***4.1.3 The BMP-4-induced $p21^{CIP1}$ and $p27^{KIP1}$ expression and $G_0/G_1$ arrest in MG63 cells is mediated by the BMPRIA/Smad5 pathway.*** The treatment of MG63 cells with BMP-4 (25 ng/ml) induced a rapid increase (within 30 min) in Smad1/5 phosphorylation, which reached a maximal level ≈5 times that of untreated controls within 1 h, and then declined but remained elevated after 24 h of treatment (Figure 4-3A). The increases in Smad1/5 phosphorylation were similar with BMP-4 concentrations of 25, 50, and 100 ng/ml, indicating that the BMP-4-induced Smad1/5 activation was dose-independent over the range tested (Figure 4-3B). The transfection of MG63 cells with BMPRIA-specific siRNA (40 nM, compared with control siRNA) abolished the BMP-4-induced Smad1/5 phosphorylation (Figure 4-3C). In contrast, BMPRIB-specific siRNA did not have inhibitory effects on the BMP-4-induced Smad1/5 phosphorylation. These results suggest that the BMP-4-induced Smad1/5 activation is mediated by BMPRIA, but not BMPRIB. BMPRIA- and BMPRIB-specific siRNAs almost totally abolished their respective receptor protein expressions (Figure 4-3C).

To investigate the role of Smad1/5 in the BMP-4-modulation of cell cycle regulatory

protein expression in the MG63 cells, the cells were transfected with Smad1- or Smad5-specific siRNA (40 nM), which reduced the expressions of the corresponding Smad proteins by ≈80% compared to that with control siRNA (Figure 4-3D), and the cells were then kept as controls or treated with BMP-4 for 48 h. The transfection with Smad5-specific siRNA (compared with control siRNA) resulted in a significant inhibition of the BMP-4-induced up-regulation of $p21^{CIP1}$ and $p27^{KIP1}$ expression (Figure 4-3D). In contrast, the transfection with Smad1-specific siRNA only inhibited the BMP-4-induced up-regulation of $p21^{CIP1}$ expression. To elucidate the importance of BMPRIA and Smad5 in modulating the BMP-4-induced $G_0/G_1$ arrest, MG63 cells were transfected with BMPRIA- or Smad5-specific siRNA (40 nM) and then kept as controls or treated with BMP-4 for 72 h. For unstimulated cells, the transfection with BMPRIA- or Smad5-specific siRNA (compared with control siRNA) did not alter their cell cycle distribution (Table 4-3). After BMP-4 treatment, the MG63 cells transfected with either BMPRIA- or Smad5-specific siRNA had a significantly lower cell percentage in the $G_0/G_1$ phases and a higher cell percentage in synthetic and $G_2$/M phases, as compared with cells transfected with the control siRNA (Table 4-3). Taken together, these results suggest that the BMP-4-induced $p21^{CIP1}$ and $p27^{KIP1}$ expression and $G_0/G_1$ arrest in MG63 cells is mediated through the BMPRIA/Smad5 pathway.

***4.1.4 ERK mediates the BMP-4-induced Smad phosphorylation in MG63 cells.*** The ERK pathway is known to regulate gene expression and cellular functions, notably cell proliferation and differentiation [121]. We investigated the role of ERK in the BMP-induced Smad signaling in MG63 cells. The MG63 cells stimulated with BMP-4 (25 ng/ml) induced a rapid increase in ERK phosphorylation within 10 min after stimulation (Figure 4-4A). This increased level of phosphorylation decreased to nearly the basal level after 30 min post-BMP-4 stimulation. The transfection of MG63 cells with BMPRIA- and BMPRIB-specific siRNA did not inhibit the BMP-4-induced ERK phosphorylation (Figure 4-4B), indicating

that the BMP-4-induced ERK activation is not mediated by the BMP receptor-signaling pathway. However, when MG63 cells were transfected with ERK-specific siRNA (40 nM), which caused 89-90% reduction in the protein expression of ERK, their BMP-4-induced Smad1/5 phosphorylation was significantly reduced, as compared with the cells transfected with control siRNA (Figure 4-4C). These results suggest that ERK mediates the BMP-4-induced Smad1/5 activation in MG63 cells.

***4.1.5 The BMP-4-induced ERK and Smad phosphorylations in MG63 cells are mediated by $\beta_3$, but not $\alpha_v$ and $\beta_1$, integrins.*** To elucidate the role of $\alpha_v$, $\beta_1$, and $\beta_3$ integrins in the BMP-4-induced ERK and Smad1/5 activation in MG63 cells, the cells were transfected with $\alpha_v$-, $\beta_1$-, and $\beta_3$-specific siRNAs (40 nM for each), and then kept as controls or stimulated with BMP-4 (25 ng/ml) for 10 and 30 min. The BMP-4-induced ERK and Smad1/5 phosphorylations were inhibited by transfections of MG63 cells with $\beta_3$-specific siRNA (compared with control siRNA), but not $\alpha_v$- and $\beta_1$-specific siRNAs, suggesting that the BMP-4-induced ERK and Smad1/5 activations are mediated by $\beta_3$, but not $\alpha_v$ and $\beta_1$, integrins (Figure 4-5A). The $\alpha_v$-specific siRNA had 60-70% blocking effects on the $\alpha_v$ integrin expression; the $\beta_1$- and $\beta_3$-specific siRNAs almost totally abolished their respective integrin expressions (Figure 4-5A). We further investigated the interaction between the integrins and the BMP receptors under the unstimulated control condition or in response to BMP-4. MG63 cells were kept as controls or treated with BMP-4 (25 ng/ml) for 10 min, and their extracts were immunoprecipitated with an antibody against BMPRIA or BMPRIB, followed by Western blot analysis with antibodies against the $\alpha_v$, $\beta_1$, and $\beta_3$ integrins. In the unstimulated cells, $\alpha_v$, $\beta_1$, and $\beta_3$ integrins showed constitutive associations with BMPRIA and BMPRIB (Figure 4-5B). The BMP-4 stimulation resulted in a significant decrease in the association of BMPRIA with the $\beta_1$ integrin, as well as BMPRIB with the $\beta_1$ and $\beta_3$

integrins. BMP-4 did not affect the association of BMPRIA with the $\alpha_v$ and $\beta_3$ integrins, as well as BMPRIB with the $\alpha_v$ integrin.

## 4.2 Tumor cell cycle arrest induced by shear stress: role of integrins and Smad

***4.2.1 Shear stress induces $G_2$/M arrest and the corresponding changes in cell cycle regulatory protein expression in tumor cells.*** The effects of shear stress on cell cycle distribution were studied in four tumor cell lines (human MG63 and Saos2 osteosarcoma cells, SCC25 oral squamous carcinoma cells, and SW1353 chondrosarcoma cells). Cells were kept as controls or subjected to shear stress (12 dynes/cm$^2$ for 24 and 48 h). Flow cytometry showed that the incubation of these tumor cells under static conditions for 24 or 48 h led to increases in the cell % in $G_0/G_1$ phases and decreases in the synthetic and/or $G_2$/M phases (Table 4-4), indicating a $G_0/G_1$ arrest. The application of shear stress to these tumor cells caused significant increases in the cell % in the $G_2$/M phases and decreases in the $G_0/G_1$ phases, as compared with cells under static conditions for the same periods.

We investigated the molecular basis of this shear effect, and the presentation is focused on human MG63 cells. The application of shear stress to MG63 cells for 24 or 48 h increased cyclin B1 and $p21^{CIP1}$ expression and decreased Cdk1 expression (Figure 4-6). The decrease in Cdk1 expression was accompanied by an increase in its tyrosine 15 phosphorylation, indicating a shear-induced decrease in Cdk1 activity [122]. Shear stress also decreased the expression of cyclins A, D1, and E, Cdk-2, -4, and -6, and $p27^{KIP1}$.

***4.2.2 Shear stress induces sustained phosphorylation of Smad1/5 in MG63 cells through BMPRIA.*** The application of shear stress to MG63 induced a rapid increase (within 10 min) in Smad1/5 phosphorylation, which reached a maximal level of ~10 times static controls within 1 h, and then declined but remained elevated after 24 h of shearing (Figure 4-7A).

The increases in Smad1/5 phosphorylation were similar with shear stresses of 2, 12, and 20 dynes/cm$^2$, indicating that the shear-induced Smad1/5 activation was shear dose-independent over the range tested (Figure 4-7B). Pre-treating MG63 cells with Noggin, a specific antagonist that binds BMPs to block their binding to the BMP receptors, did not inhibit the shear-induced Smad1/5 phosphorylation; hence, the shear-activation of Smad1/5 was not mediated by BMPs (Figure 4-7C). As a positive control, the pre-treatment of MG63 cells with Noggin did cause an inhibition of the Smad1/5 phosphorylation induced by BMP-4.

To study the types of BMP receptors responsible for the shear-activation of Smad1/5, MG63 cells were transfected with BMPRIA- or BMPRIB-specific small interfering RNA (siRNA, 40 nM), which reduced the expressions of the corresponding receptor proteins by ~2/3 of those with control siRNA (Figure 4-7D), and the cells were then exposed to shear stress for 30 min. The shear-induced Smad1/5 phosphorylation was abolished by the BMPRIA-specific siRNA, but was not inhibited by the BMPRIB-specific siRNA (Figure 4-7E).

***4.2.3 Shear-induced $G_2$/M arrest in tumor cells is mediated by Smad1/5.*** MG63 cells were transfected with Smad1- or Smad5-specific siRNA (40 nM), which caused 80-90% reductions in expressions of the corresponding Smads (Figure 4-8A), and the cells were then kept under static conditions or exposed to flow for 48 h. Under static conditions, the MG63 cells transfected with Smad1- or Smad5-specific siRNA (compared to control siRNA, 40 nM) did not alter their cell cycle distribution (Table 4-5). Following shear stress, the MG63 cells transfected with either Smad5- or Smad1-specific siRNA had a significantly higher cell % in the $G_0/G_1$ and synthetic phases and a lower cell % in the $G_2$/M phases, as compared to cells transfected with control siRNA (Table 4-5). The transfection with Smad1- or Smad5-specific siRNA (compared to control siRNA) resulted in a significant inhibition of the shear-induced up-regulation of Cdk1 tyrosine 15 phosphorylation (Figure 4-8B).

Smad1-specific and Smad5-specific siRNAs have some differential actions, with the former inhibiting the shear-induced cyclin A down-regulation and cyclin B1 up-regulation, and the latter inhibiting the shear-induced $p21^{CIP1}$ up-regulation. Neither Smad-specific siRNA had significant effects on the shear-induced changes of the other cell cycle regulatory proteins (Figure 4-8B).

***4.2.4 $\alpha_v\beta_3$ and $\beta_1$ integrins mediate shear-induced Smad1/5 phosphorylation and $G_2$/M arrest in tumor cells.*** MG63 cells were pre-treated with RGDS (Arg-Gly-Asp-Ser), which blocks the cell-ECM interaction mediated by the integrin-recognition sequence RGD (Arg-Gly-Asp) on ECM proteins, or with specific antibodies against the $\alpha_v\beta_3$ and $\beta_1$ integrins, and were then kept under static condition or exposed to flow for 30 min. Pre-treatment with RGDS and integrin antibodies significantly inhibited the shear-induced Smad1/5 phosphorylation, as compared to cells pre-treated with control RGES or IgG (Figure 4-9A). The inhibition of shear-induced Smad1/5 phosphorylation by blocking the $\alpha_v\beta_3$ and $\beta_1$ integrins was substantiated by the transfection of cells with $\alpha_v$-, $\beta_1$-, and $\beta_3$-specific siRNAs (40 nM for each), which also showed significant inhibitory effects on the shear-induced Smad1/5 phosphorylation (Figure 4-9B). The $\alpha_v$- and $\beta_3$-specific siRNAs had 50-60% blocking effects on their respective integrin expressions; the $\beta_1$-specific siRNA almost totally abolished the $\beta_1$ expression (Figure 4-9C). The transfection with dominant-negative mutants of Shc and focal adhesion kinase (FAK) (compared to empty vector pcDNA3) did not have significant effects on the shear-induced Smad1/5 phosphorylation (Figure 4-9D). The pre-treatment of MG63 cells with specific antibodies against $\alpha_v\beta_3$ and $\beta_1$ did not affect the cell cycle distribution in static control cells, but under shear stress they caused a significant increase in the cell % in $G_0/G_1$ or synthetic phases and a decrease in the $G_2$/M phases (Table 4-5).

***4.2.5 Shear stress inhibits MG63 cell differentiation and Runx2 binding activity in the nucleus.*** MG63 cells were kept as controls or exposed to shear stress for 1, 3, 6, and 24 h, and their expression of differentiation marker genes, i.e., osteocalcin (OCN) and alkaline phosphatase (ALP), was examined. Shearing for 6 and 24 h resulted in significant decreases in OCN and ALP gene expression (Figure 4-10A). These effects of shearing on MG63 cells were substantiated by the shear-induced decreases in the activities of ALP and luciferase, which were determined by the transfection of cells with OCN-Luc containing the promoter region of the human OCN gene in front of the luciferase gene (Figure 4-10B). Since the promoter regions of the OCN and ALP genes contain the Runx2 binding domain that is responsible for the modulation of these genes [65], we tested whether shear stress regulates Runx2 binding activity in MG63 cells. The results of the electrophoretic mobility shift assay (EMSA), obtained by incubating nuclear protein extracts of the cells with oligonucleotides corresponding to the Runx2 binding sequences of the OCN promoter, showed that shear stress caused a sustained decrease in the Runx2 binding activity over the period tested (24 h of flow) (Figure 4-10C). As a positive control, the treatment of MG63 cells with BMP-4 for 6 and 24 h induced Runx2 binding activity. The specificity of this binding for Runx2 was shown by its abolition by co-incubation of the nuclear proteins with 20-fold unlabeled oligonucleotides, and by the supershifting in the gel mobility after the pre-incubation of nuclear proteins with an antibody to Runx2.

***4.2.6 $\alpha_v\beta_3$ and $\beta_1$ integrins and Smad5 mediate the shear-induced inhibition of MG63 cell differentiation.*** The transfection of MG63 cells with specific siRNA of Smad5 (compared with control siRNA), but not Smad1, inhibited the shear-induced down-regulation of OCN and ALP expressions (Figure 4-10D) and nuclear binding activity for Runx2 (Figure 4-10E). The shear-induced down-regulation of OCN and ALP expressions was also inhibited by pre-treating MG63 cells with a specific antibody against $\alpha_v\beta_3$ or $\beta_1$ (Figure 4-10F). These

results suggest that the shear-induced inhibition of MG63 cell differentiation was mediated by $\alpha_v\beta_3$ and $\beta_1$ integrins through Smad5, but not Smad1.

**Table 4-1. BMP-4 induces $G_0/G_1$ arrest in human MG63 and mouse MC3T3-E1 osteoblast-like cells.**

| Cell type | Control | | | BMP4 | | |
|---|---|---|---|---|---|---|
| | % of cells (mean ± SEM ) | | | % of cells (mean ± SEM ) | | |
| | $G_0/G_1$ | Synthetic | $G_2/M$ | $G_0/G_1$ | Synthetic | $G_2/M$ |
| **MG63** | | | | | | |
| 0 h | 43.6±2.5% | 30.0±1.5% | 26.4±4.0% | – | – | – |
| 24 h | 62.9±2.3% * | 22.6±2.0% | 14.5±4.2% | 61.9±1.1% | 24.9±3.5% | 13.2±2.4% |
| 48 h | 67.6±1.2% * | 16.0±0.1% * | 16.4±1.3% | 80.4±2.6% # | 9.9±1.1% # | 9.7±1.5% # |
| 72 h | 76.8±2.1% * | 11.9±0.8% * | 11.3±1.3% * | 91.4±1.0% # | 4.7±1.3% # | 3.9±0.4% # |
| 96 h | 79.2±1.6% * | 11.3±0.1% * | 9.5±1.6% * | 94.3±1.2% # | 2.6±0.8% # | 3.1±0.4% # |
| **MC3T3-E1** | | | | | | |
| 0 h | 49.8±3.4% | 32.6±4.9% | 17.6±1.5% | – | – | – |
| 24 h | 55.5±1.8% | 25.1±3.8% | 19.4±2.1% | 60.2±2.5% | 19.6±0.7% | 20.2±1.8% |
| 48 h | 66.4±1.0% * | 17.7±2.2% * | 15.9±1.2% | 77.7±3.5% # | 9.7±0.7% # | 12.6±2.8% |
| 72 h | 74.8±0.8% * | 14.4±1.7% * | 10.8±0.9% * | 90.1±0.2% # | 5.8±0.5% # | 4.1±0.3% # |
| 96 h | 80.3±0.9% * | 10.6±1.0% * | 9.1±1.9% * | 95.4±1.8% # | 2.2±0.7% # | 2.4±1.1% # |

Two different osteoblast-like cell lines, i.e., human MG63 and mouse MC3T3-E1 cells, were kept as controls or treated with BMP-4 (25 ng/ml) for 24 h, 48 h, 72 h, and 96 h. The cells were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in $G_0/G_1$, synthetic, or $G_2/M$ phases of the cell cycle. Data are mean ± SEM from three independent experiments. *, $P < 0.05$ *vs*. unstimulated control cells at 0 h; #, $p < 0.05$ *vs.* unstimulated control cells at the corresponding time; –, no sample.

**Table 4-2. BMP-4-induced $G_0/G_1$ arrest in MG63 cells is dose-independent.**

| Condition | % of cells (mean ± SEM ) | | |
|---|---|---|---|
| | $G_0/G_1$ | Synthetic | $G_2/M$ |
| **Control** | | | |
| 0 h | 60.2±3.3% | 20.3±3.0% | 19.5±0.4% |
| 96 h | 79.3±1.3% * | 11.8±1.6% * | 8.9±0.3% * |
| **BMP-4 (96 h)** | | | |
| 10 ng/ml | 89.0±0.4% # | 5.4±1.0% # | 5.6±0.6% # |
| 25 ng/ml | 92.1±0.2% # | 3.8±0.1% # | 4.1±0.4% # |
| 50 ng/ml | 92.8±0.4% # | 3.4±0.2% # | 3.8±0.2% # |
| 100 ng/ml | 93.6±0.4% # | 3.3±0.5% # | 3.1±0.1% # |

MG63 cells were kept as controls or treated with BMP-4 at concentrations of 10, 25, 50, and 100 ng/ml for 96 h. The cells were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in $G_0/G_1$, synthetic, or $G_2/M$ phases of the cell cycle. Data are mean ± SEM from three independent experiments. *, $P < 0.05$ *vs.* unstimulated control cells at 0 h; #, $p < 0.05$ *vs.* unstimulated control cells at 96 h.

**Table 4-3. BMP4-induced $G_0/G_1$ arrest in MG63 cells is mediated by BMPRIA and Smad5.**

| siRNA | Control (72 h) | | | BMP-4 (72 h) | | |
|---|---|---|---|---|---|---|
| | % of cells (mean ± SEM ) | | | % of cells (mean ± SEM ) | | |
| | $G_0/G_1$ | Synthetic | $G_2/M$ | $G_0/G_1$ | Synthetic | $G_2/M$ |
| **siCL** | 78.9±1.7% | 12.9±0.6% | 8.2±1.1% | 91.5±1.0% * | 4.3±0.3% * | 4.2±0.7% * |
| **siRIA** | 75.0±3.5% | 11.8±1.6% | 13.2±1.9% | 76.5±3.8% # | 12.5±1.3% # | 11.0±2.5% # |
| **siSmad5** | 80.3±0.6% | 10.7±1.1% | 9.0±1.6% | 78.0±0.8% # | 11.9±0.8% # | 10.1±1.6% # |

MG63 cells were transfected with control siRNA (siCL) or specific siRNA of BMPRIA (siRIA) or Smad5 (siSamd5) (40 nM) for 48 h, and then were kept as controls or treated with BMP-4 (25 ng/ml) for 72 h. The cells were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in $G_0/G_1$, synthetic, or $G_2/M$ phases of the cell cycle. Data are mean ± SEM from three independent experiments. *, $P < 0.05$ *vs.* unstimulated control cells; #, $P < 0.05$ *vs.* cells transfected with control siRNA.

**Table 4-4. Shear stress induces a $G_2$/M cell cycle arrest in tumor cells.**

| Cell type | Control | | | Shear | | |
|---|---|---|---|---|---|---|
| | % of cells (mean ± SEM ) | | | % of cells (mean ± SEM ) | | |
| | $G_0/G_1$ | Synthetic | $G_2$/M | $G_0/G_1$ | Synthetic | $G_2$/M |
| **MG63** | | | | | | |
| 0h | 58.4±7.2% | 31.1±2.8% | 10.5 ±1.2% | – | – | – |
| 24h | 75.7±5.4% | 16.9±2.0%* | 7.4 ±1.1% | 64.4±7.3% | 15.9±1.2% | 19.7±2.4%# |
| 48h | 84.5±4.7%* | 9.0±1.3%* | 6.5 ±1.1%* | 65.2±4.9%# | 8.2±0.9% | 26.6±3.4%# |
| **Saos2** | | | | | | |
| 0h | 65.9±5.2% | 14.6±1.7% | 19.5 ±2.1% | – | – | – |
| 24h | 82.6±3.7%* | 9.0±0.8%* | 8.4 ±0.9%* | 50.9±5.3%# | 22.2±3.2%# | 26.9±1.5%# |
| 48h | 86.5±6.1%* | 8.8±0.6%* | 4.7 ±0.9%* | 51.7±6.5%# | 12.7±2.1% | 35.6±4.2%# |
| **SCC25** | | | | | | |
| 0h | 43.9±3.2% | 39.9±2.8% | 16.2 ±1.3% | – | – | – |
| 24h | 61.3±2.3%* | 19.9±1.1%* | 18.8 ±1.2% | 54.7±2.6% | 17.9±1.5% | 27.4±1.4%# |
| 48h | 64.3±4.8%* | 18.6±2.5%* | 17.1 ±1.5% | 50.8±2.2%# | 17.4±2.1% | 31.8±2.9%# |
| **SW1353** | | | | | | |
| 0h | 52.9±3.8% | 32.9±2.4% | 14.2 ±2.1% | – | – | – |
| 24h | 65.3±5.8% | 26.7±2.3% | 8.0 ±1.0%* | 53.1±3.9% | 16.4±2.1%# | 30.5±2.3%# |
| 48h | 66.7±2.3%* | 23.6±1.7%* | 9.7 ±0.9%* | 52.2±2.7%# | 11.5±0.8%# | 36.3±4.3%# |

Four different tumor cell lines, i.e., human MG63 and Saos2 osteosarcoma cells, SCC25 oral squamous carcinoma cells, and SW1353

chondrosarcoma cells, were kept in static conditions as controls or subjected to a shear stress of 12 dynes/cm$^2$ for 24 h and 48 h. The cells were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in $G_0/G_1$, synthetic, or $G_2$/M phases of the cell cycle. Data are mean±SEM from three independent experiments. *$P < 0.05$ *vs*. static control cells at 0 h. #$p < 0.05$ *vs*. static control cells at the corresponding time. –: no sample.

**Table 4-5. Shear-induced $G_2$/M arrest in tumor cells is mediated by $\alpha_v\beta_3$ and $\beta_1$ integrins and Smad1/5.**

| siRNA and Antibody | Control (48 h) | | | Shear (48 h) | | |
|---|---|---|---|---|---|---|
| | % of cells (mean ± SEM ) | | | % of cells (mean ± SEM ) | | |
| | $G_0/G_1$ | Synthetic | $G_2$/M | $G_0/G_1$ | Synthetic | $G_2$/M |
| **siCL** | 89.2±3.8% | 5.7±0.7% | 5.1±1.2% | 65.9±5.1%* | 2.3±0.5%* | 31.8±4.2%* |
| **siSmad1** | 86.8±4.9% | 6.0±1.0% | 7.2±1.2% | 67.1±6.1%* | 16.2±1.3%*,# | 16.7±2.4%*,# |
| **siSmad5** | 90.0±3.2% | 4.3±0.7% | 5.7±1.0% | 82.7±7.3%# | 12.9±0.8%*,# | 4.4±1.1%# |
| **IgG** | 83.1±4.9% | 7.2±1.4% | 9.7±0.8% | 63.2±8.1%* | 6.7±1.1% | 30.1±2.6%* |
| **Anti-$\alpha_v\beta_3$** | 86.1±5.3% | 5.5±0.9% | 8.4±1.0% | 85.9±6.8%# | 8.6±1.3% | 5.5±1.1%# |
| **Anti-$\beta_1$** | 83.2±7.6% | 7.4±0.8% | 9.4±0.8% | 74.8±9.8% | 13.9±2.2%*,# | 11.3±1.5%# |

MG63 cells were (1) transfected with control siRNA (siCL) or specific siRNA of Smad1 (siSmad1) or Smad5 (siSamd5) (40 nM for each) for 48 h, or (2) pre-treated with control IgG or a specific antibody against $\alpha_v\beta_3$ (Anti-$\alpha_v\beta_3$) or $\beta_1$ (Anti-$\beta_1$) (10 μg/mL for each) for 2 h, and then were kept under static conditions (Control) or exposed to flow (Shear) for 48 h. The cells were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in $G_0/G_1$, synthetic, or $G_2$/M phases of the cell cycle. Data are mean±SEM from three independent experiments. *$P < 0.05$ *vs.* static control cells. #$p < 0.05$ *vs.* the cells transfected with control siRNA or pre-treated with control IgG.

**Figure 4-1**

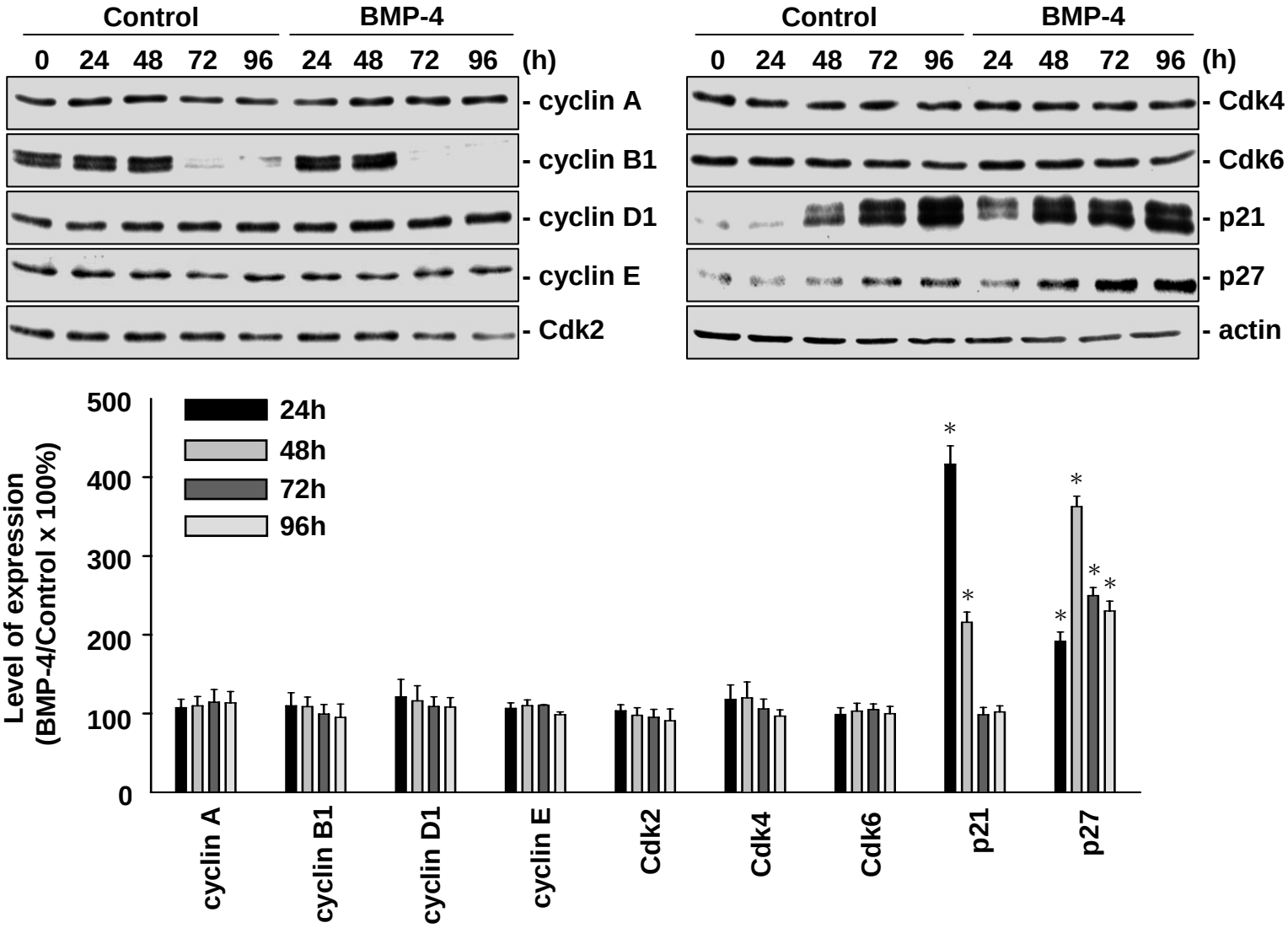

Control
BMP-4
0 24 48 72 96 24 48 72 96 (h)
- cyclin A
- cyclin B1
- cyclin D1
- cyclin E
- Cdk2
Control
BMP-4
0 24 48 72 96 24 48 72 96 (h)
- Cdk4
- Cdk6
- p21
- p27
- actin
Level of expression (BMP-4/Control x 100%)
500
400
300
200
100
0
24h
48h
72h
96h
cyclin A
cyclin B1
cyclin D1
cyclin E
Cdk2
Cdk4
Cdk6
p21
p27

**Figure 4-1. BMP-4 regulates expression of cell cycle regulatory proteins in MG63 cells.** MG63 cells were kept as controls or stimulated with BMP-4 (25 ng/ml) for 24, 48, 72, and 96 h. Expression of cell cycle regulatory proteins was determined by Western blot analysis. Data are mean ± SEM from three independent experiments. *, $P < 0.05$ *vs.* unstimulated control cells.

**Figure 4-2**

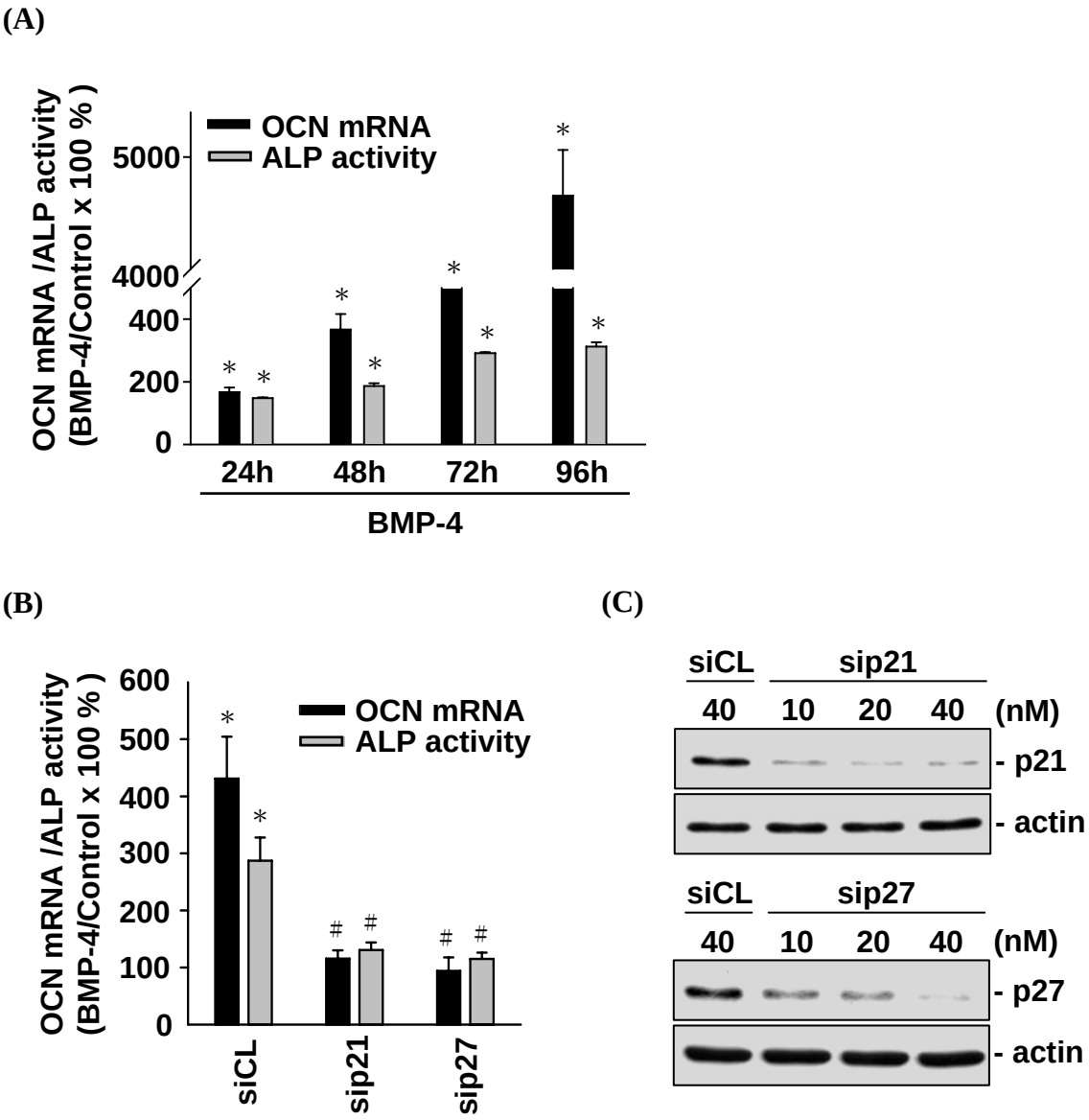



**Figure 4-2. BMP-4-induced MG63 cell differentiation is mediated by $p21^{CIP1}$ and $p27^{KIP1}$.** (*A*) MG63 cells were kept as controls or stimulated with BMP-4 (25 ng/ml) for 24, 48, 72, and 96 h, and their OCN mRNA expression and ALP activity were determined by real-time PCR and ALP activity assay, respectively. (*B*) MG63 cells were transfected with control siRNA (siCL) or a specific siRNA of $p21^{CIP1}$ (sip21) or $p27^{KIP1}$ (sip27) (40 nM for each) for 48 h, and then treated with BMP-4 for 72 h. (*C*) MG63 cells were transfected with p21- or p27-specific siRNA at indicated concentrations for 48 h, and their $p21^{CIP1}$ or $p27^{KIP1}$ protein expression was determined by Western blot analysis. Data in (*A*) and (*B*) are mean ± SEM from three to four independent experiments. Results in (*C*) are representative of three independent experiments with similar results. *, $P < 0.05$ *vs.* unstimulated control cells (*A*). #, $P < 0.05$ *vs.* cells transfected with control siRNA (*B*).

**Figure 4-3**

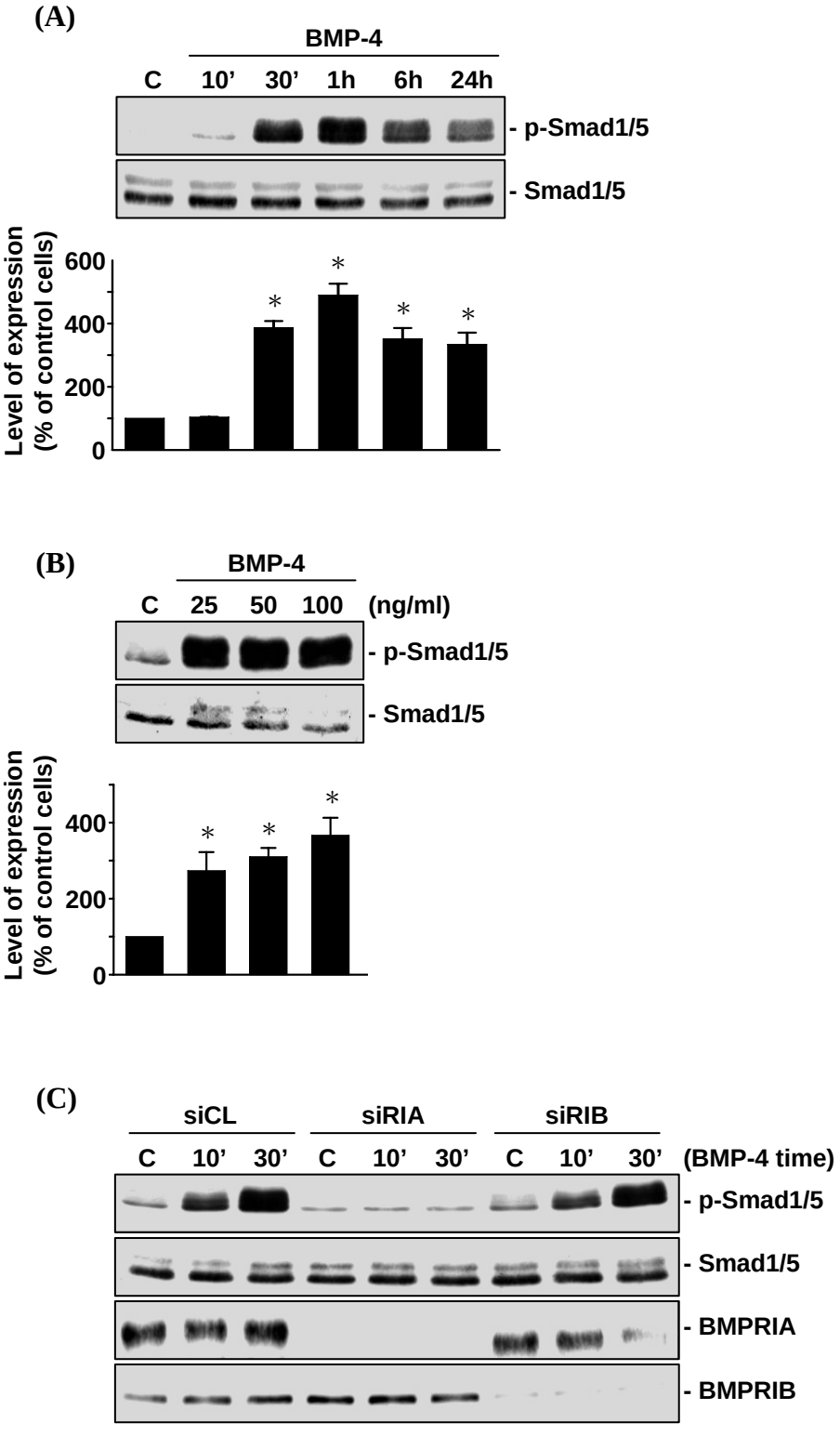

(A)
BMP-4
C
10'
30'
1h
6h
24h
- p-Smad1/5
- Smad1/5
Level of expression (% of control cells)
600
400
200
0
*
(B)
BMP-4
C
25
50
100
(ng/ml)
- p-Smad1/5
- Smad1/5
Level of expression (% of control cells)
400
200
0
*
(C)
siCL
siRIA
siRIB
C
10'
30'
C
10'
30'
C
10'
30'
(BMP-4 time)
- p-Smad1/5
- Smad1/5
- BMPRIA
- BMPRIB

**Figure 4-3**

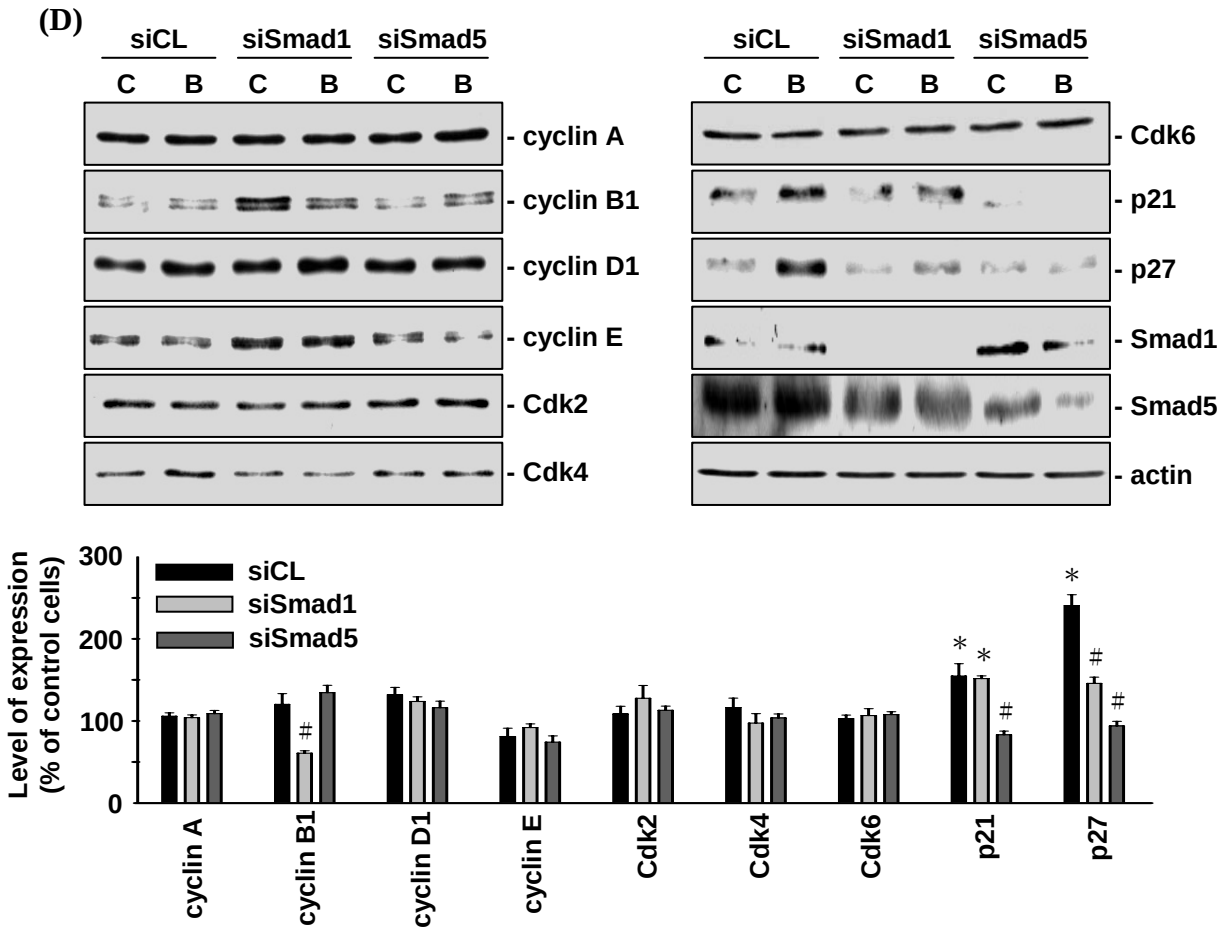



**Figure 4-3. BMP-4-induced p21$^{CIP1}$ and p27$^{KIP1}$ expressions are mediated by BMPRIA/Smad5 in MG63 cells.** (*A*) MG63 cells were kept as controls (C) or treated with BMP-4 (25 ng/ml) for 10 min (10'), 30 min (30'), 1 h, 3 h, 6 h, and 24 h, and their Smad1/5 phosphorylations were determined by Western blot analysis. (*B*) MG63 cells were treated with BMP-4 of 25, 50, and 100 ng/ml for 1 h. (*C*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of BMPRIA (siRIA) or BMPRIB (siRIB) (40 nM for each) for 48 h, and then were kept as controls or treated with BMP-4 for 10 or 30 min. (*D*) MG63 cells were transfected with control siRNA or specific siRNA of Smad1 (siSmad1) or Smad5 (siSmad5) for 48 h (40 nM for each), and then were kept as controls (C) or treated with BMP-4 (B) for 48 h. Data in (*A*), (*B*), and (*D*) are mean ± SEM from three independent experiments, and are presented as percentage changes in band density from control cells normalized to Smad1/5 (*A* and *B*) or actin (*D*) protein levels. Results in (*C*) are representative of three independent experiments with similar results. *, $P < 0.05$ *vs.* unstimulated control cells. #, $P < 0.05$ *vs.* cells transfected with control siRNA.

**Figure 4-4**

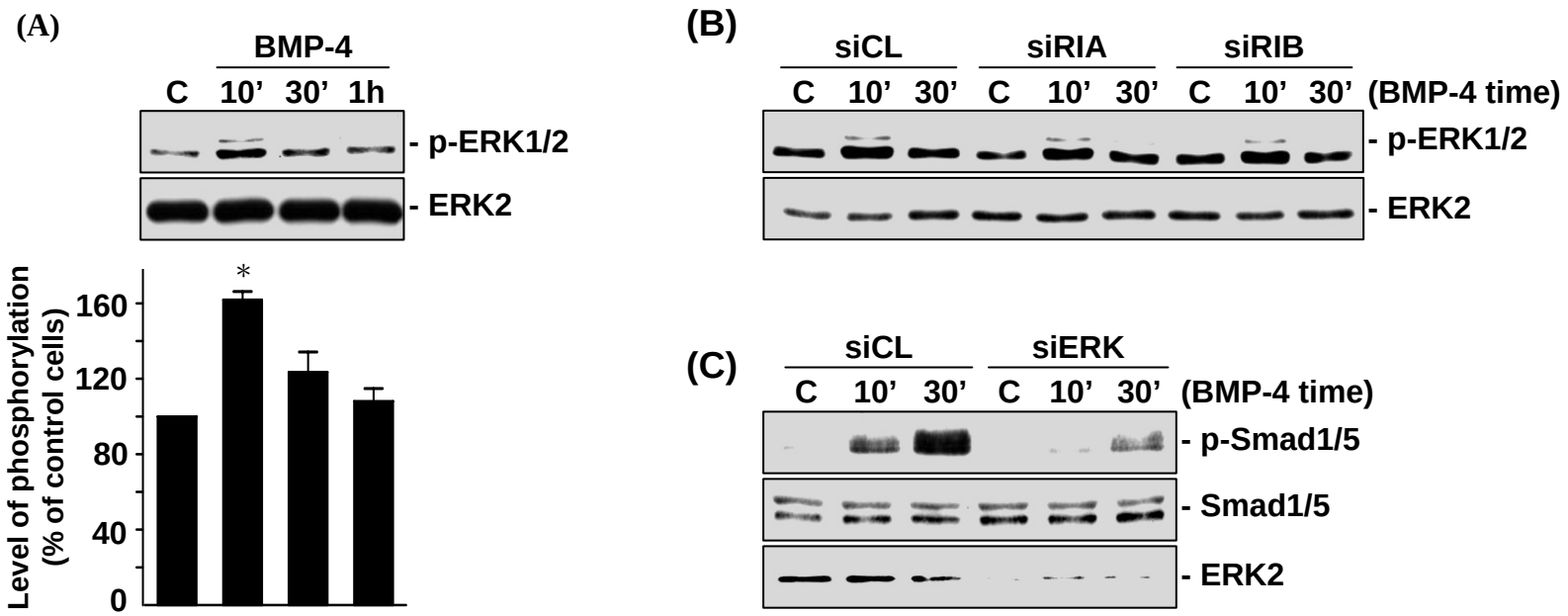



**Figure 4-4. BMP-4-induced Smad1/5 activation is mediated by ERK in MG63 cells.** (*A*) MG63 cells were kept as controls (C) or treated with BMP-4 (25 ng/ml) for 10 min (10'), 30 min (30'), and 1 h, and their ERK phosphorylations were determined by Western blot analysis. Data are mean ± SEM from three independent experiments, and are presented as percentage changes in band density from control cells normalized to ERK2 protein levels. *, $P < 0.05$ *vs.* unstimulated control cells. (*B* and *C*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of BMPRIA (siRIA) or BMPRIB (siRIB) (*B*), or ERK (siERK) (*C*) (40 nM for each) for 48 h, and then were kept as controls (C) or treated with BMP-4 for 10 (10') or 30 (30') min. The phosphorylations of ERK (*B*) or Smad1/5 (*C*) were determined by Western blot analysis. Results are representative of three independent experiments with similar results.

**Figure 4-5**

**(A)**

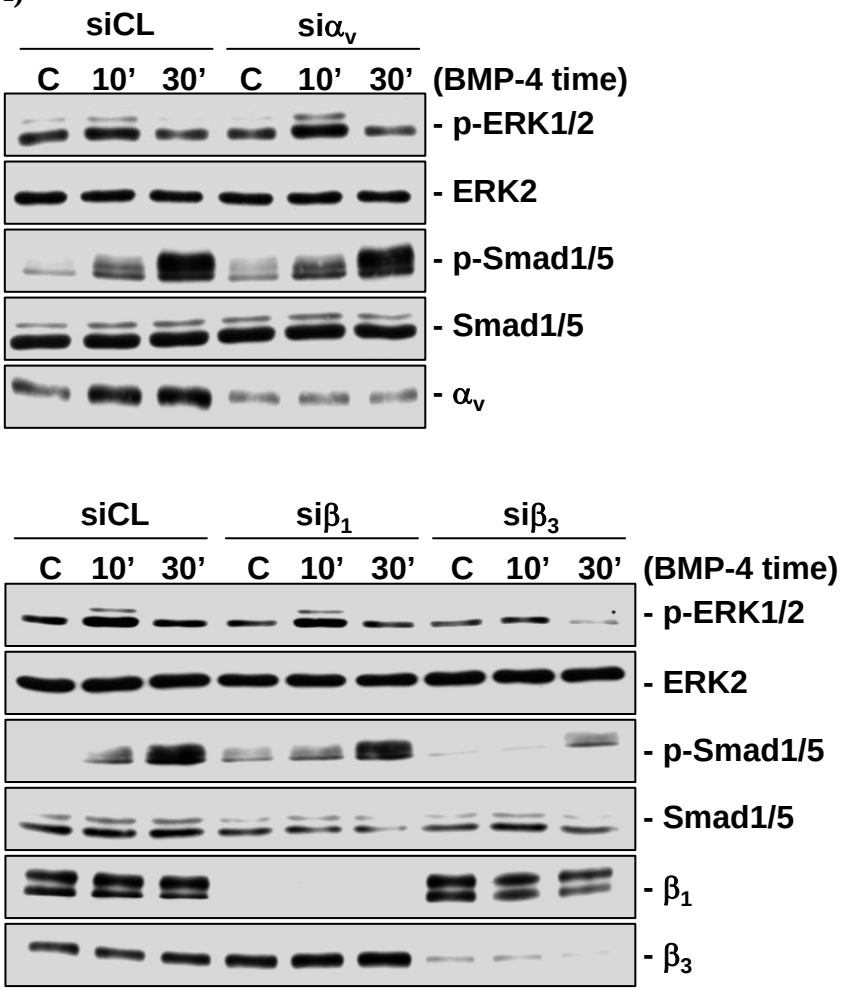

siCL
siαv
C 10' 30' C 10' 30' (BMP-4 time)
- p-ERK1/2
- ERK2
- p-Smad1/5
- Smad1/5
- αv
siCL
siβ1
siβ3
C 10' 30' C 10' 30' C 10' 30' (BMP-4 time)
- p-ERK1/2
- ERK2
- p-Smad1/5
- Smad1/5
- β1
- β3

**Figure 4-5**

**(B)**

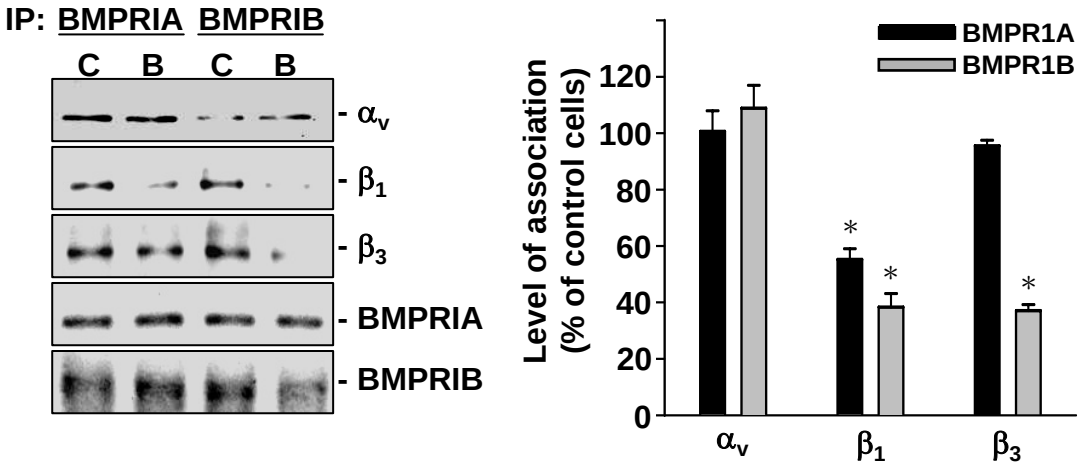



**Figure 4-5. BMP-4-induced ERK and Smad1/5 activations are mediated by $\beta_3$ integrin in MG63 cells.** (*A*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of $\alpha_v$ (si$\alpha_v$), $\beta_1$ (si$\beta_1$), or $\beta_3$ (si$\beta_3$) integrin (40 nM for each) for 48 h, and then were kept as controls (C) or treated with BMP-4 (25 ng/ml) for 10 (10') or 30 (30') min. The phosphorylations of ERK and Smad1/5 were determined by Western blot analysis. Results are representative of three independent experiments with similar results. (*B*) MG63 cells were kept as controls (C) or treated with BMP-4 (B; 25 ng/ml) for 10 min. The associations of BMPRIA and BMPRIB with $\alpha_v$, $\beta_1$, and $\beta_3$ integrins were determined by immunoprecipitation assay and Western blot analysis, as described in Materials and Methods. The amounts of intergrin-BMP receptor complexes in BMP-4-stimulated cells are presented as band densities (normalized to BMP receptors) relative to those in control cells. The results are mean ± SEM from three independent experiments. *, $P < 0.05$ *vs.* unstimulated control cells.

**Figure 4-6**

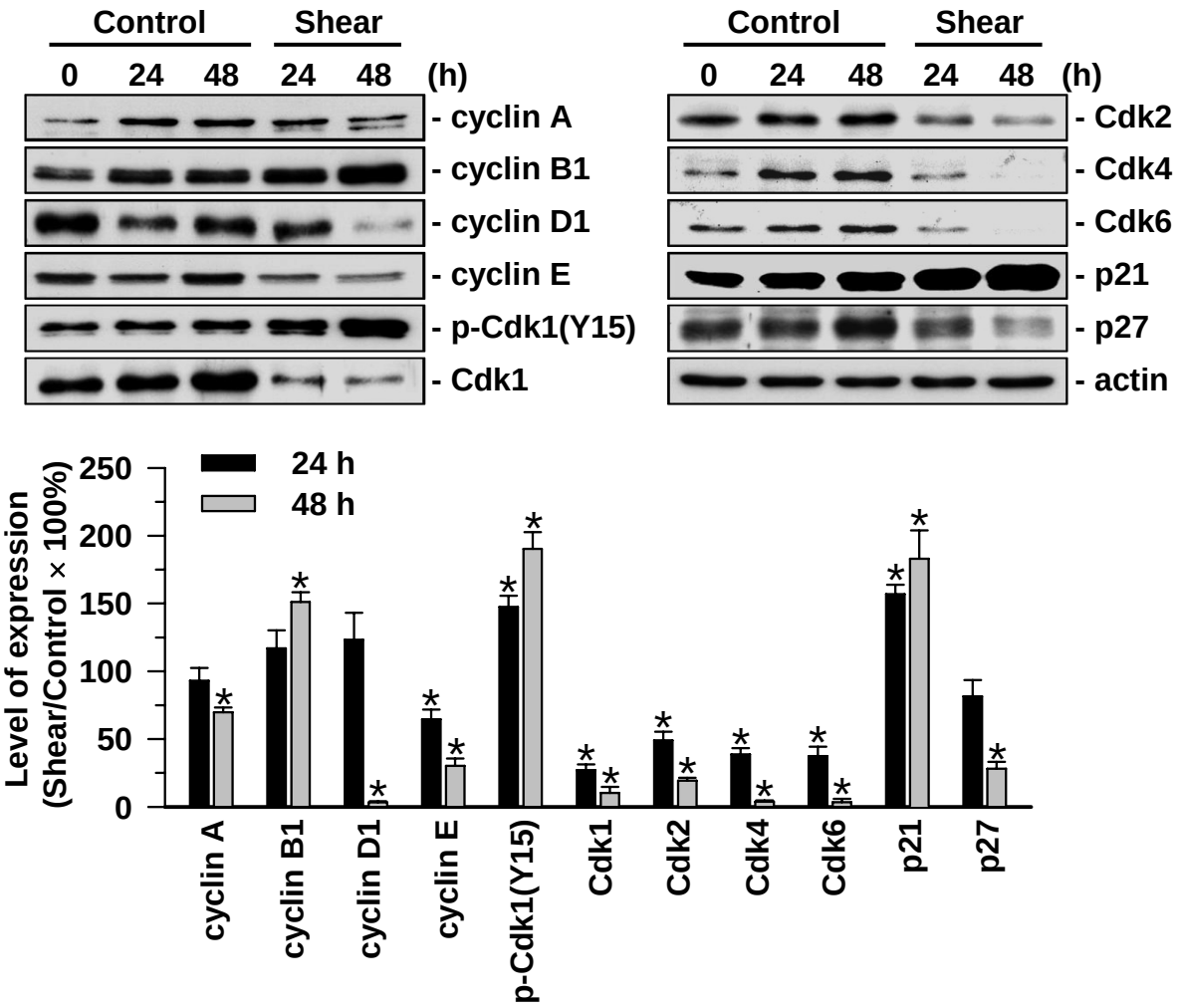



**Figure 4-6. Shear stress regulates expressions of cell cycle regulatory proteins in MG63 cells.** MG63 cells were kept as controls or subjected to shear stress (12 dynes/cm$^2$) for 24 and 48 h. Expression of cell cycle regulatory proteins was determined by Western blot analysis. Data are mean±SEM from three independent experiments. *$P < 0.05$ *vs*. static control cells.

**Figure 4-7**

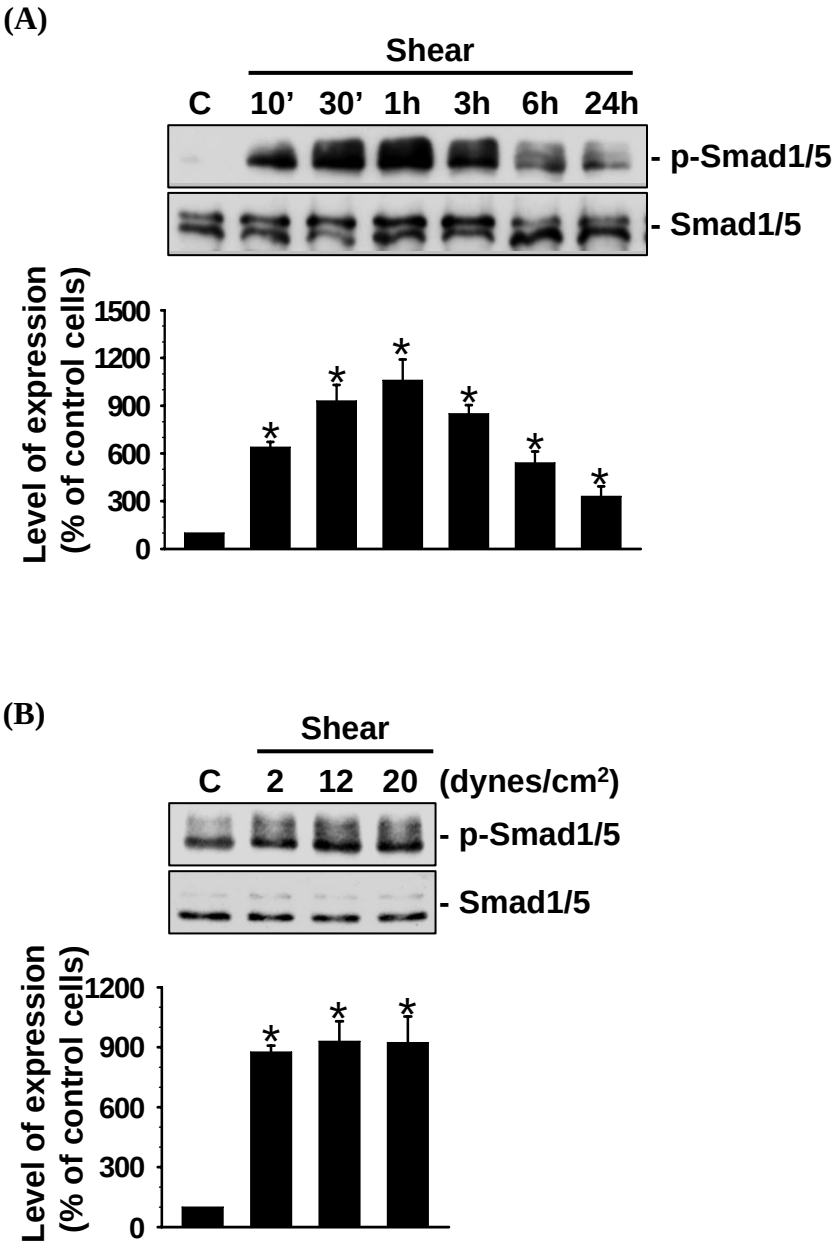

(A)
Shear
C
10'
30'
1h
3h
6h
24h
- p-Smad1/5
- Smad1/5
Level of expression
(% of control cells)
1500
1200
900
600
300
0
(B)
Shear
C
2
12
20
(dynes/cm2)
- p-Smad1/5
- Smad1/5
Level of expression
(% of control cells)
1200
900
600
300
0

**Figure 4-7**

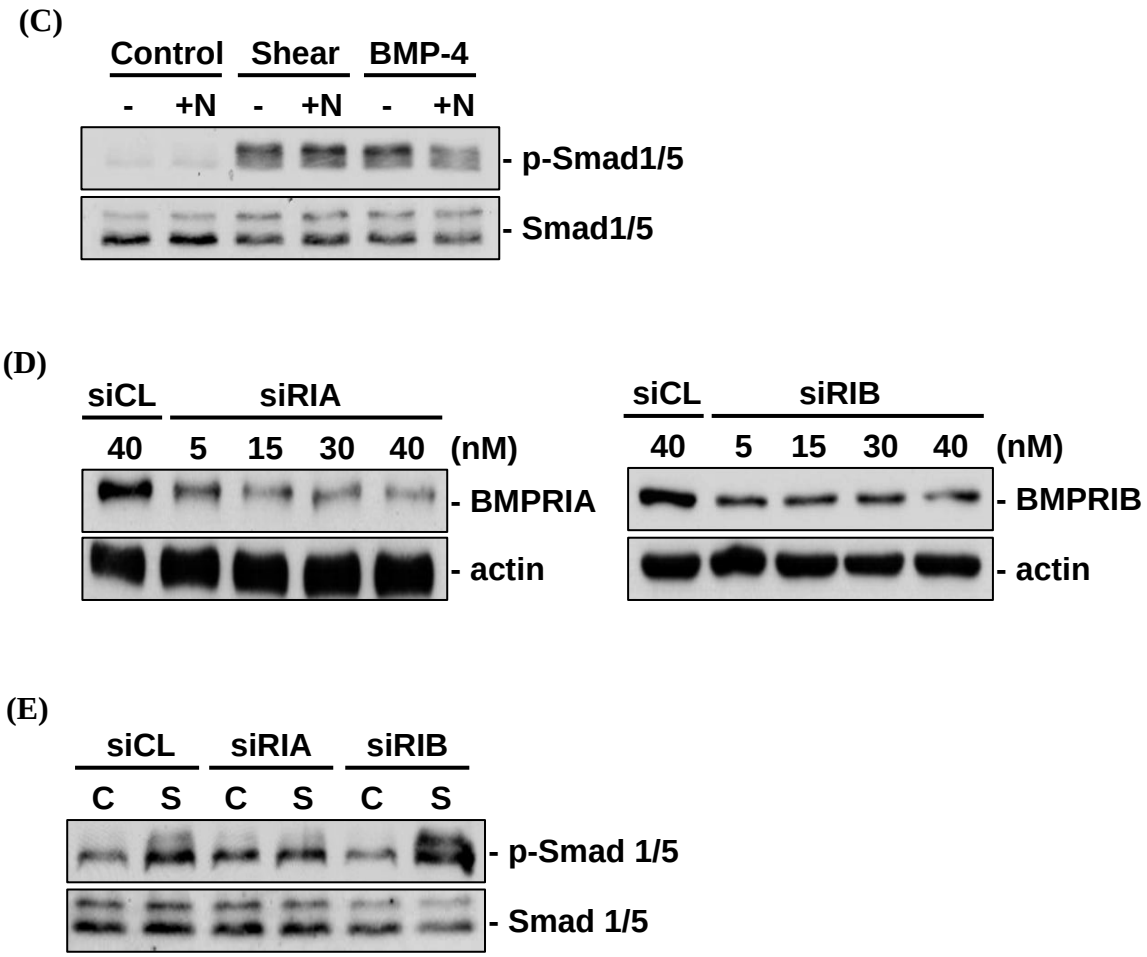



**Figure 4-7. Shear stress induces sustained phosphorylation of Smad1/5 in MG63 cells through BMPRIA.** (*A*) MG63 cells were kept as controls (C) or subjected to shear stress (12 dynes/cm$^2$) for 10', 30', 1 h, 3 h, 6 h, and 24 h, and their Smad 1/5 phosphorylation was determined by Western blot analysis. (*B*) MG63 cells were exposed to shear stress of 2, 12, and 20 dynes/cm$^2$ for 30 min. (*C*) MG63 cells were kept as controls (-) or pre-treated with Noggin (100 ng/mL) for 1 h (+N), and then subjected to flow or BMP-4 (100 ng/mL) for 30 min. (*D*) MG63 cells were transfected with siRNAs at various concentrations (5, 15, 30, and 40 nM) and their BMPRIA and BMPRIB protein expressions were examined by Western blot analysis. (*E*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of BMPRIA (siRIA) or BMPRIB (siRIB) (40 nM each) for 48 h, and then kept as controls (C) or exposed to flow (S) for 30 min. Data in (*A*) and (*B*) are mean±SEM from three independent experiments, and presented as % changes in band density from control cells normalized to Smad1/5 protein levels. The results in (*C*)-(*E*) are representative of triplicate experiments with similar results. * $P < 0.05$ *vs.* static control cells.

**Figure 4-8**

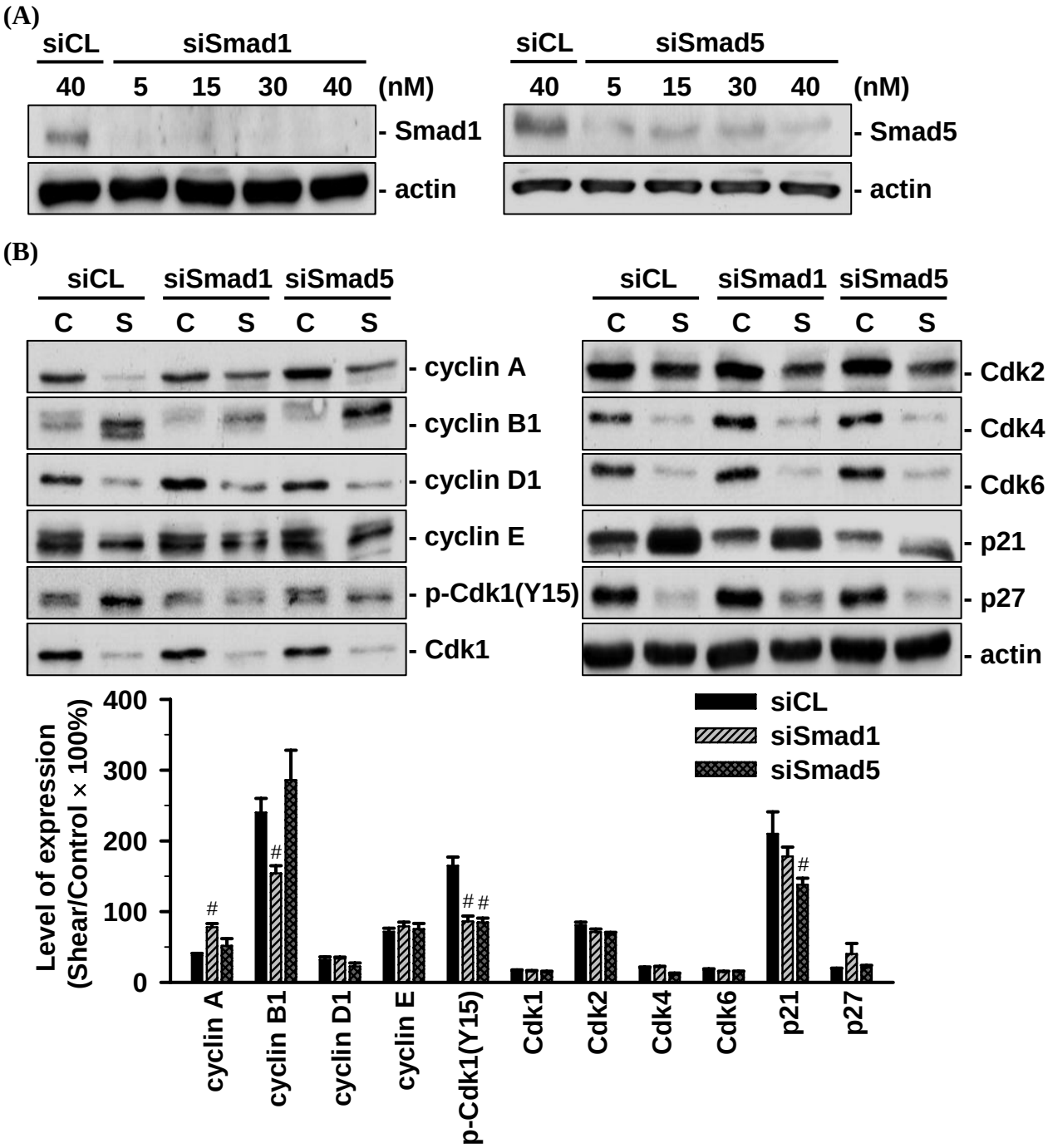



**Figure 4-8. Shear-induced $G_2$/M arrest in MG63 cells is mediated by Smad1/5.** (*A*) MG63 cells were transfected with Smad1- or Smad5-specific siRNA at 5, 15, 30, or 40 nM for 48 h, and Smad1 and Smad5 protein expressions were determined by Western blot analysis. Results are representative of triplicate experiments with similar results. (*B*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of Smad1 (siSmad1) or Smad5 (siSamd5) (40 nM each), and then kept under static conditions (C) or exposed to flow (S) for 48 h. The expression of cell cycle regulatory proteins was determined by Western blot analysis. Data are mean±SEM from three independent experiments, and are presented as % changes in band density from control cells normalized to Smad1/5 protein levels. # $P < 0.05$ *vs.* cells transfected with control siRNA.

**Figure 4-9**

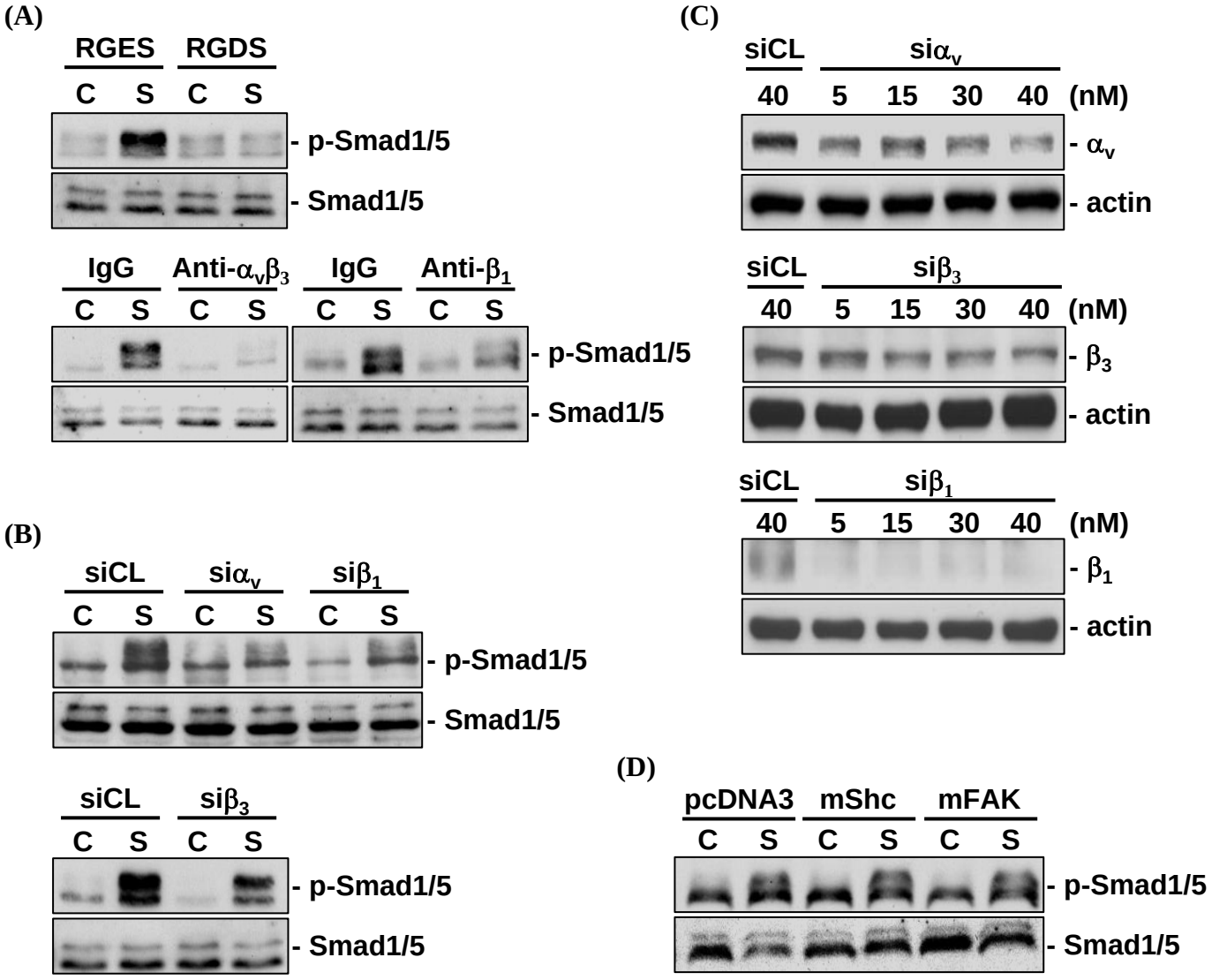
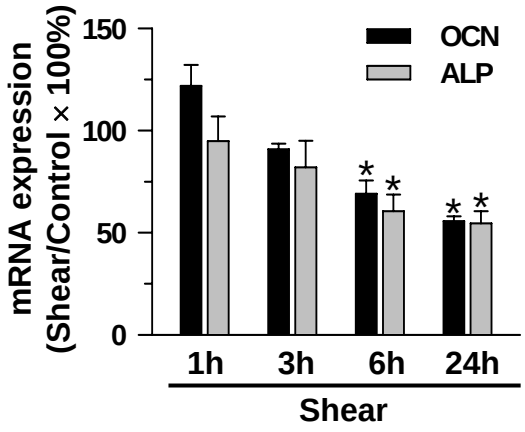


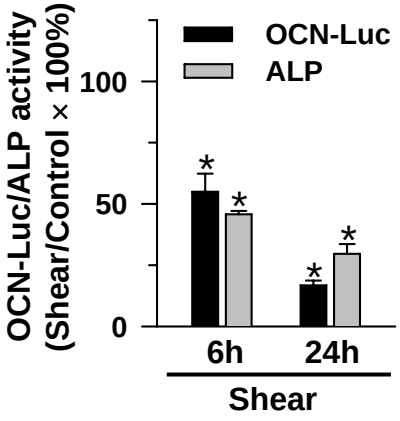

**Figure 4-9. Shear-induced Smad1/5 activations are mediated by $\alpha_v\beta_3$ and $\beta_1$ integrins in MG63 cells.** (*A*) MG63 cells were pre-treated with RGDS peptides (500 μg/mL) or specific antibodies against $\beta_1$ (Anti-$\beta_1$) or $\alpha_v\beta_3$ (Anti-$\alpha_v\beta_3$) for 2 h (10 μg/mL each), and kept under static conditions (C) or subjected to shear stress (12 dynes/cm$^2$) (S) for 30 min. As controls, the cells were pre-treated with RGES (500 μg/mL) and non-specific control IgG (10 μg/mL). (*B*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of $\alpha_v$ (si$\alpha_v$), $\beta_1$ (si$\beta_1$), or $\beta_3$ (si$\beta_3$) (40 nM each) for 48 h prior to exposure to flow. (*C*) MG63 cells were transfected with specific siRNAs of integrins at 5, 15, 30, or 40 nM, and their integrin protein expressions were examined. (*D*) MG63 cells were transfected with 3 μg of Shc-SH2 (mShc), FAK(F397Y) (mFAK), or pcDNA3 empty vector for 48 h prior to exposure to flow (S). Results are representative of triplicate experiments with similar results.

**Figure 4-10**

**(A)**




**(B)**




**(C)**

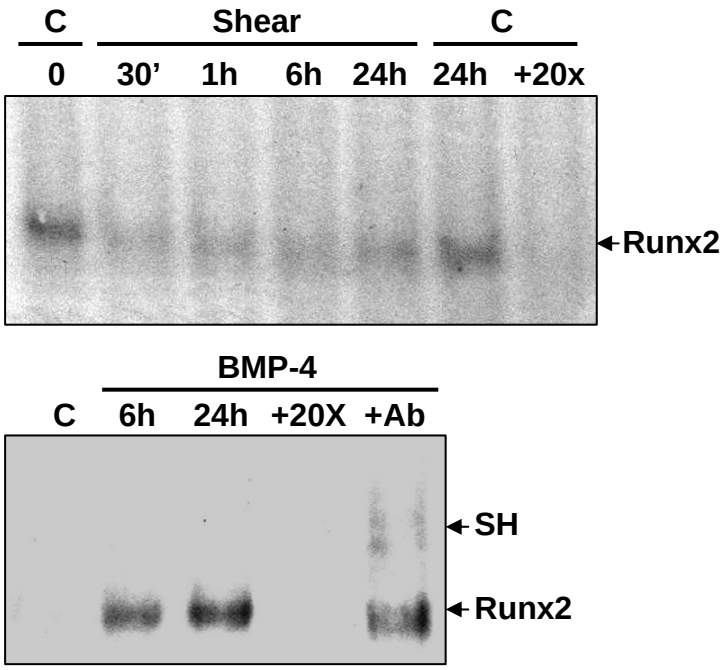


**Figure 4-10**

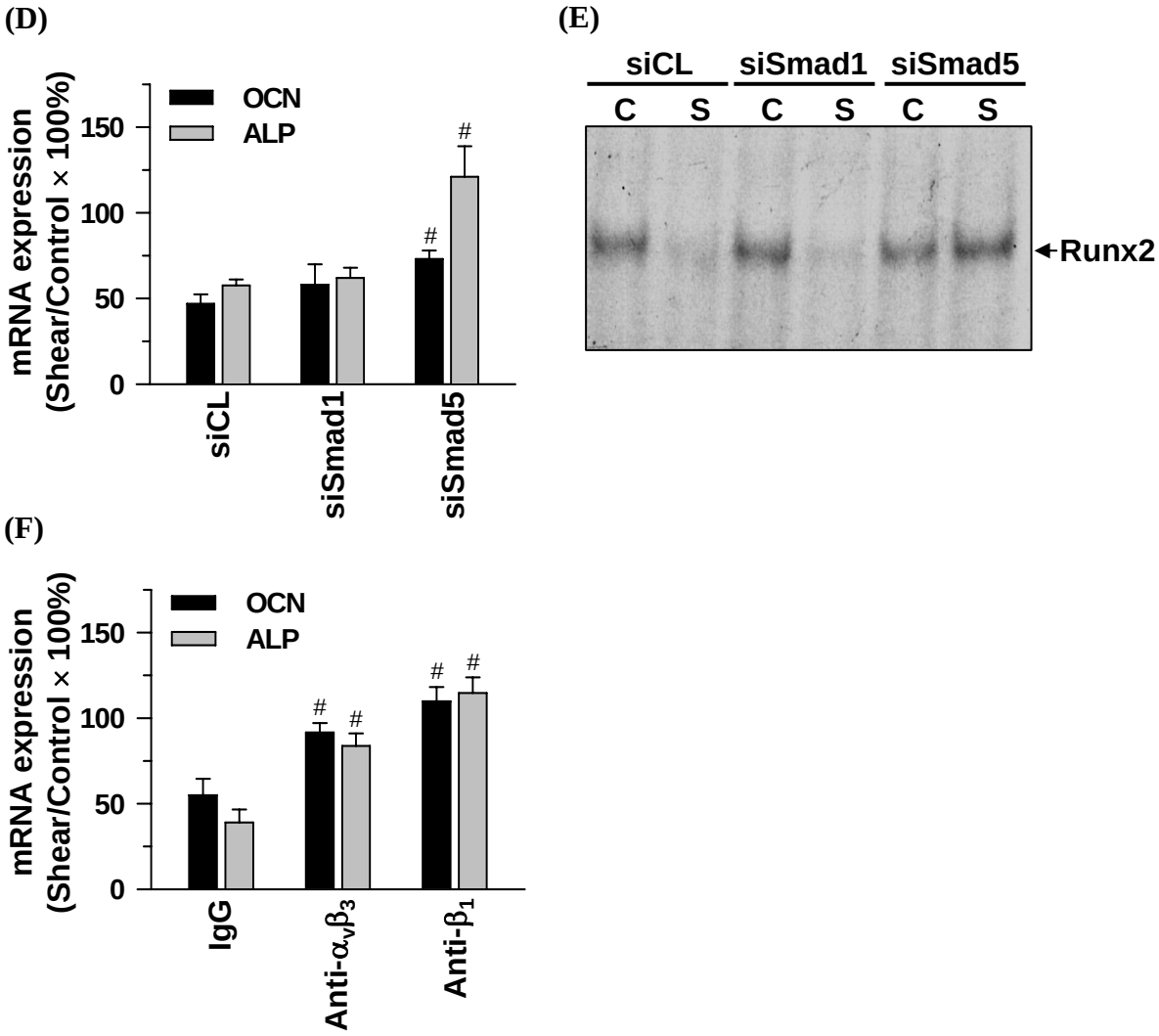



**Figure 4-10. MG63 cell differentiation and Runx2 binding activity in the nucleus were inhibited by shear stress acting through $\alpha_v\beta_3$ and $\beta_1$ integrins and Smad5.** MG63 cells were kept as controls (C) or subjected to shear stress (12 dynes/cm$^2$) for 30' to 24 h, as indicated (*A*, *B*, and *C*). The cells were transfected with empty vector control PSR$\alpha$ or OCN-Luc (1 $\mu$g/mL each) (*B*), transfected with siRNA (siCL for control, or specific siRNA of Smad1 or Smad5) (40 nM each) (*D* and *E*) for 48 h, or pre-treated with control IgG or a specific antibody against $\alpha_v\beta_3$ or $\beta_1$ (10 $\mu$g/mL each) for 2 h (*F*), and then were kept under static conditions or subjected to shear stress for 6 and 24 h (*B*) or 24 h (*D-F*). The OCN and ALP mRNA expressions (*A*, *D*, and *E*), OCN promoter and ALP activities (*B*), and Runx2-DNA binding activity (*C* and *E*) were determined by real-time PCR, luciferase and ALP activity assays, and EMSA, respectively. In (*C*), total nuclear extracts of cells and $^{32}$P-labeled oligonucleotides containing human OCN Runx2 binding sites were used. Nuclear extracts were pre-incubated with 20-fold excess unlabeled oligonucleotides (+20×). As positive controls, MG63 cells were treated with BMP-4 (100 ng/mL) for 6 and 24 h (*C*). Nuclear extracts pre-incubated with the Runx2 antibody (+Ab) show a super shift band (SH). Results in (*A*, *B*, *D*, and *F*) are mean±SEM from three to four independent experiments. Results in (*C* and *E*) are representative of two or three independent experiments with similar results. *$P < 0.05$ *vs.* static control cells (*A* and *B*). # $P < 0.05$ *vs.* control treatments (*D, F*).

# CHAPTER V

# DISCUSSION

## 5.1 Bone Morphogenetic Protein-4 Induces Osteoblast $G_0/G_1$ Arrest and Differentiation via Increased Expression of $p21^{CIP1}$ and $p27^{KIP1}$: Role of integrins, extracellular signal-regulated kinase, and Smad

There is accumulating evidence that proliferating mammalian progenitor cells must exit cell cycle in order to differentiate into mature cells [69, 72-73]. BMPs are a family of signaling molecules derived from bone, which induce the differentiation of mesenchymal-type cells into osteoblasts and hence allow bone formation [9-11, 30]. The aim of the present study was to investigate the role of BMP-4 in the cell cycle distribution in osteoblast-like cells and the underlying mechanisms. In a series of systematic studies, we have characterized the mechanisms by which BMP-4 regulates the cell cycle, and hence differentiation, in osteoblast-like cells, through specific BMP receptors, integrins, MAPKs, and downstream Smad signaling and cell cycle regulatory proteins as summarized in Figure 5-1. Our study has generated the following findings. First, this work shows that BMP-4 induces $G_0/G_1$ arrest in human MG63 and mouse MC3T3-E1 osteoblast-like cells, with concomitant increases in the expression of Cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. This BMP-4-induction of $p21^{CIP1}$ and $p27^{KIP1}$ expression is prerequisite for the differentiation of these osteoblast-like cells. Second, the BMP-4-induced $G_0/G_1$ arrest and increased expression of $p21^{CIP1}$ and $p27^{KIP1}$ is mediated by BMPRIA, but not BMPRIB, through Smad5. Third, our study showed that the BMP-4-induced activation of Smad1/5 is mediated by $\beta_3$ integrin through ERK, with the existence of a $\beta_3$-BMPRIA heteromeric complex in BMP-4-stimulated cells. Thus, our findings provide insights into the molecular mechanisms by which BMP-4 regulates the cell cycle and differentiation in osteoblast-like cells.

It is known that entry into and progression through the cell cycle is regulated by different cell cycle regulatory proteins, including cyclin-Cdk complexes and Cdk inhibitors, which facilitate the transition between different phases of the cell cycle [72]. The role of BMP-4 in

regulating the cell cycle distribution and the regulatory protein expression in osteoblasts has not been fully clarified. Wong et al. [123] demonstrated that BMP-2 inhibits proliferation of human aortic smooth muscle cells *via* increased an expression of $p21^{CIP1}$. Franzen et al. [124] demonstrated that BMP-7 induces cell cycle arrest in anaplastic thyroid carcinoma cells *via* the increased expression of $p21^{CIP}$ and $p27^{KIP}$ and decreased activity of Cdk2 and Cdk6. A recent study by Jeffery et al. [125] demonstrated that BMP-4 inhibits the proliferation of human fetal lung fibroblasts and promotes their differentiation into myofibroblasts through Smad1 and $p21^{CIP1}$. These results suggest that $p21^{CIP1}$ and $p27^{KIP1}$ may serve as the downstream targets of BMP signaling and contribute to the anti-proliferative and pro-differentiation effects of BMPs. In the present study, we found that BMP-4 induces the increased expression of $p21^{CIP1}$ and $p27^{KIP1}$ in MG63 cells. Further support for the involvement of $p21^{CIP1}$ and $p27^{KIP1}$ in the pro-differentiation effect of BMP-4 was provided by the findings that the transfection of cells with $p21^{CIP1}$- and $p27^{KIP1}$-specific siRNAs abolished the BMP-4-induced expression or activity of the differentiation markers OCN and ALP. Moreover, our results showed that the BMP-4-induced expression of both $p21^{CIP1}$ and $p27^{KIP1}$ was mediated by Smad5, inasmuch as transfection of cells with Smad5-specific siRNA abolished the BMP-4-induction of both $p21^{CIP1}$ and $p27^{KIP1}$. Recent studies showed that TGF-β-specific Smad3 may form complexes with transcription cofactors E2F and p107 that bind to the promoter of the c-myc gene and hence repress its expression [126-129]. Whether BMP-specific Smad5 can also form complexes with E2F and p107 that directly bind to the promoters of $p21^{CIP1}$ and $p27^{KIP1}$, and consequently increase their expression, remains to be determined. It is noted that incubation of MG63 and MC3T3-E1 cells under static conditions for 48 h, 72 h, or 96 h also led to $G_0/G_1$ arrest (Table 4-1). This cell contact-induced $G_0/G_1$ arrest was also accompanied by the increase in $p21^{CIP1}$ and $p27^{KIP1}$ expression in unstimulated control cells (Figure 4-1). Thus, $p21^{CIP1}$ and $p27^{KIP1}$ may also be

involved in the cell contact-induced cell cycle arrest in unstimulated control cells. Our findings indicate the importance of $p21^{CIP1}$ and $p27^{KIP1}$ in regulating cell cycle in osteoblast-like cells, under the unstimulated control condition or in response to BMP-4.

BMPs exert their effects *via* binding two types of serine/threonine kinase receptors; the type II receptor is a constitutively active kinase, which transphosphorylates type I receptors (i.e., BMPRIA and BMPRIB) upon ligand binding, leading to activation of the downstream signals, Smads [29-35]. BMPRIA and BMPRIB have been shown to exert differential biological effects [29-32]. It has been reported that BMPRIA is critical for BMP-2-induced adipocyte differentiation, whereas BMPRIB was shown to be responsible for BMP-2-induced osteoblast differentiation and apoptosis [31]. These results suggest that BMPRIA and BMPRIB may transmit different signals to bone-derived mesenchymal progenitors and play critical roles in both the specification and differentiation of osteoblasts and adipocytes. However, additional studies found that there is no functional difference between BMPRIA and BMPRIB in modulating the BMP-induced osteoblast differentiation [32]. By using BMPRIA- and BMPRIB-specific siRNAs, our results showed that the BMP-4-induced cell cycle arrest *via* Smad activation in MG63 cells was mediated by BMPRIA, but not BMPRIB. The discrepancy in the biological effects of BMPRIA and BMPRIB between our present results and the previous studies may be due to the different cell types used in the various experimental systems.

It has been reported that osteoblasts express $\alpha_v$-, $\beta_1$- and $\beta_3$-containing integrins at the focal adhesion sites, and that these integrins play important roles in regulating the signaling and functions in osteoblasts [51-54]. Using specific siRNA, our present study demonstrated that $\beta_3$, but not $\beta_1$ and $\alpha_v$, integrins exert regulatory effects on the BMP-4-induced Smad signaling in MG63 cells. Numerous mechanisms have been shown to be attributable to the

regulatory effects of integrins on osteoblasts [130-131]. However, the mechanism by which integrins mediate the BMP-eliciting signaling in osteoblasts remains unclear. There is evidence that integrins may be cooperative with the receptors of several growth factors, including insulin receptor and platelet-derived growth factor-β receptor, to form integrin-receptor heteromeric complexes that mediate downstream signaling cascades [102]. A recent study by Lai and Cheng [54] showed that $\alpha_v$ and $\beta_1$ integrins can interact with BMP-2 receptors in BMP-2-stimulated osteoblasts, and that these integrins may serve as anchors for BMP-2 receptors to facilitate the activation of downstream signaling cascades. Our coimmunoprecipitation experiments, using an antibody against BMPRIA or BMPRIB followed by Western blot analysis using an antibody against the $\beta_3$ integrin, showed that the $\beta_3$ integrin can form heteromeric complexes with BMPRIA and BMPRIB in the unstimulated MG63 cells. However, the BMP-4 stimulation resulted in a significant inhibition in the formation of the $\beta_3$-BMPRIB complex, but did not influence the formation of the $\beta_3$-BMPRIA complex. The existence of the $\beta_3$-BMPRIA heteromeric complex in the BMP-4-stimulated cells provides the possibility for close crosstalk between the $\beta_3$ integrin and BMPRIA, which may exert synergistic effects at the levels of downstream signaling pathways and gene expression in response to BMP-4.

In addition to Smad, our present results in MG63 cells showed that BMP-4 induces a transient activation of ERK, a member of the MAPK family activated through Ras. This BMP-4-induced activation of ERK was not mediated through the BMP receptors, inasmuch as BMPRIA- and BMPRIB-specific siRNAs did not inhibit the BMP-4-induced ERK phosphorylation. However, the BMP-4-induced Smad1/5 activation was mediated by ERK, as evidenced by the inhibition of the BMP-4-induced Smad1/5 phosphorylation by ERK-specific siRNA. The role of the MAPK pathway in BMP signaling is less clear. The

activation of ERK was shown to be involved in the regulation of the BMP-2-induced differentiation of mesenchymal C3H10T1/2 cells into the osteoblast phenotype [46]. Recent studies demonstrated that the ERK pathway can be activated by integrins and consequently contributes to the integrins-mediated osteoblast differentiation [131-132]. In the present study, the role of integrins in the BMP-4-induced ERK and hence Smad1/5 activation was evidenced by the blockage of the BMP-4-induced ERK and Smad1/5 phosphorylation by transfection of the cells with $\beta_3$-specific siRNA. Thus, our findings demonstrated that BMP-4 induces Smad1/5 phosphorylation through BMPRIA, and that this BMP-4-induced Smad1/5 phosphorylation is mediated by the $\beta_3$ integrin through ERK. It remains to be resolved whether the $\beta_3$ integrin/ERK pathway plays a direct or permissive role in mediating the BMP-4-induced signaling and gene expression in osteoblasts.

### 5.2 Tumor cell cycle arrest induced by shear stress: roles of integrins and Smad

The aim of this study was to investigate the role of shear stress in the cell cycle distribution in tumor cells and the underlying mechanisms. In a series of systematic studies, we have characterized the mechanisms by which shear stress regulates the cell cycle in tumor cells through specific integrins and their modulation of BMP receptor-specific Smads, as summarized in Figure 5-2. Our study has generated the following new findings. (1) This work shows for the first time that shear stress induces a $G_2$/M cell cycle arrest in several types of tumor cells. This shear-induced $G_2$/M arrest is associated with corresponding changes in the expression and activity of $G_2$/M regulatory proteins. (2) Our study showed a novel signaling pathway from integrins to BMPRIA-specific Smads (independent of BMPs) that modulates the expression of cell cycle regulatory proteins, and hence the cell cycle distribution, in tumor cells in response to mechanical forces. (3) In addition to regulating cell cycle, shear stress can inhibit the differentiation of osteosarcoma cells by inhibiting Runx2 binding activity in the nucleus. These shear-induced inhibitions in Runx2 activity

and cell differentiation are mediated by the $\alpha_v\beta_3$ and $\beta_1$ integrins through Smad5, but not Smad1 (Figure 5-2). Thus, our findings provide new insights into the molecular mechanisms by which flow-induced shear force regulates the cell cycle and differentiation in tumor cells.

Cell cycle arrest is a major cellular response to DNA damage preceding the decision to repair or die. In cell cycle progression, increasing accumulation of cyclin D-Cdk4/6 and cyclin A/E-Cdk2 complexes regulates the transition through the $G_1$ and synthetic phases. The subsequent $G_2$-M transition is mainly regulated by cyclin B - Cdk1 activity, which is suppressed by Cdk1 phosphorylation of tyrosine 15 during $G_2$/M [122]. Recent studies showed that tumor cell cycle arrest at $G_2$/M is associated with an increase in cyclin B1 [133]. In addition to inhibiting Cdk-2, -4, and -6 activities and promoting the $G_1$-synthetic phase transition, $p21^{CIP1}$ can accumulate in nuclei near the $G_2$/M boundary and can cause a transient block in the late $G_2$ phase [72]. These reports suggest that a decrease in Cdk1 activity and an increase in cyclin B1 and $p21^{CIP1}$ expression can promote cell cycle arrest at the $G_2$/M phases. In the present study, we found that shear stress caused a sustained decrease in Cdk1 activity (due to Cdk1 tyrosine 15 phosphorylation) and an increase in cyclin B1 and $p21^{CIP1}$ expression; these shear-induced modulations of $G_2$/M regulatory protein expression and activity could contribute to the shear-induced $G_2$/M arrest in tumor cells.

BMPRIA-specific Smad signaling has been shown to suppress tumorigenesis at gastric epithelial junctional zones [134]. Mice with a conditional inactivation of BMPRIA or over-expressing a BMP antagonist in the intestine develop intestinal tumors. Mice with Smad4-deficient T cells develop epithelial tumors in the intestinal tract [135]. These results suggest that BMPRIA-specific Smad signaling may play a tumor-suppressive role. By using specific siRNAs for different BMP receptors (i.e., BMPRIA and BMPRIB), our study showed

that the shear-induced Smad1/5 activation was mediated by BMPRIA. This shear-induced Smad1/5 activation was not due to the autocrine effect of BMPs released from sheared cells, since it was not inhibited by the specific BMP antagonist Noggin. Our results also demonstrated that the shear-induced BMPRIA-specific Smad1/5 activation contributes to the shear-induced $G_2$/M arrest in tumor cells by modulating the expression and activity of $G_2$/M regulatory proteins. Thus, shear stress may play a tumor-suppressive role by inducing a $G_2$/M cell cycle arrest in tumor cells through the BMP-independent activation of the BMPRIA-specific Smad signaling pathway.

The blockade of the shear-induced Smad1/5 phosphorylation by using the RGD peptide, specific integrin antibodies, and specific siRNAs provides evidence that the $\alpha_v\beta_3$ and $\beta_1$ integrins act as upstream signaling molecules for the shear-induced Smad1/5 activation in tumor cells. The detailed mechanism by which $\alpha_v\beta_3$ and $\beta_1$ mediate shear-induced Smad1/5 activation remains unclear. It is not likely that Shc and FAK participate in the modulation of shear-induced Smad1/5 activation by integrins, since the transfection of MG63 cells with their dominant-negative mutants did not inhibit the Smad1/5 response to shear. There is evidence that integrins may be cooperative with the receptors of several growth factors, including insulin receptor and platelet-derived growth factor-β receptor, to form integrin-receptor heteromeric complexes that mediate downstream signaling cascades [102]. The mechanism by which integrins mediate the BMPRIA-specific Smad signaling in tumor cells in response to shear stress warrants further investigation.

In addition to being an important regulatory transcription factor for mesenchymal lineage cell differentiation [65], Runx2 has been shown to activate the expression of adhesion proteins, matrix metalloproteinases, and angiogenic factors in tumor cells and promote tumor metastasis [136]. Inhibition of Runx2 in MDA-MB-231 cells transplanted to bone inhibits

tumorigenesis and prevents osteolysis [137]. These results suggest that the inhibition of Runx2 activity may have therapeutic potential against tumor development. Our demonstration that shear stress induces a down-regulation of Runx2 activity in human osteosarcoma cells supports the notion that shear stress may act as a tumor suppressor by inhibiting Runx2 activity in tumor cells, thereby inhibiting tumor growth and metastasis.

It is difficult to directly measure interstitial flow velocities and the resulting shear stresses *in vivo* due to their slowness and heterogeneity [92-93]. Measured velocities have been reported to vary between 0.1 and 4.0 μm/s [138-139]. Using a mathematical model of interstitial pressure-fluid flow, Jain et al. [140] showed that the interstitial fluid velocity in tumors is nearly zero in the center and increases rapidly in the periphery. In the present study, we studied one carcinoma line (i.e., human SCC25 oral squamous carcinoma cells) and three sarcoma lines (i.e., human MG63 and Saos2 osteosarcoma cells and SW1353 chondrosarcoma cells). These sarcoma cells are originated from tumors in bone and cartilage, which are both tissues whose formation and development are highly influenced by the mechanical microenvironment. Since the interstitial flow-induced shear stress on bone cells in response to mechanical loading has been found to be 8-30 dynes/cm$^2$ *in vivo* [141], we used a shear stress level of 12 dynes/cm$^2$, which would be more relevant to the periphery of tumors [140], to investigate the role of shear stress in modulating tumor cell signaling, gene expression, cell cycle, and differentiation. Our results suggest that mechanical forces play a significant role in modulating tumor cell responses and functions (cell cycle and differentiation). Normalization of tumor vasculature has emerged as an important concept in anti-angiogenic therapy, and this may alter the interstitial flow environment and enhance drug delivery [140, 142]. Our findings suggest that the response of tumor cells to changes in interstitial flow-induced shear force should be considered in the management of the disease.

Another implication of our study is the potential role of shear stress in tumor metastasis. Tumor cells leaving a primary tumor are subjected to shear stress in blood and lymphatic vessels. There is evidence that tumor cell metastasis occurs mainly to some specific tissues/organs, e.g., liver [143], but not to others, e.g., heart and large arterial wall [144]. It is possible that such region-specificity for tumor metastasis is related to the different shear stress/flow patterns. The present findings suggest that the tissues/organs receiving a laminar shear stress with a large forward component (12 dynes/cm$^2$) would result in the $G_2$/M arrest of the metastatic tumor cells; such $G_2$/M arrest would facilitate the removal of tumor cells by the immune system. There is evidence that a disturbed flow with a very low net shear stress has effects on intracellular signaling and cell cycle that are opposite to those of a shear stress with a large forward flow [145-146]. Therefore, it is possible that tumor cells in regions receiving a disturbed/low shear flow would not undergo $G_2$/M arrest and their metastasis/invasion would thus be promoted. Thus, different flow patterns may have differential actions on tumor cell metastasis or invasion, and the effect of a disturbed flow on tumor cell cycle deserves further investigation.

**Figure 5-1**

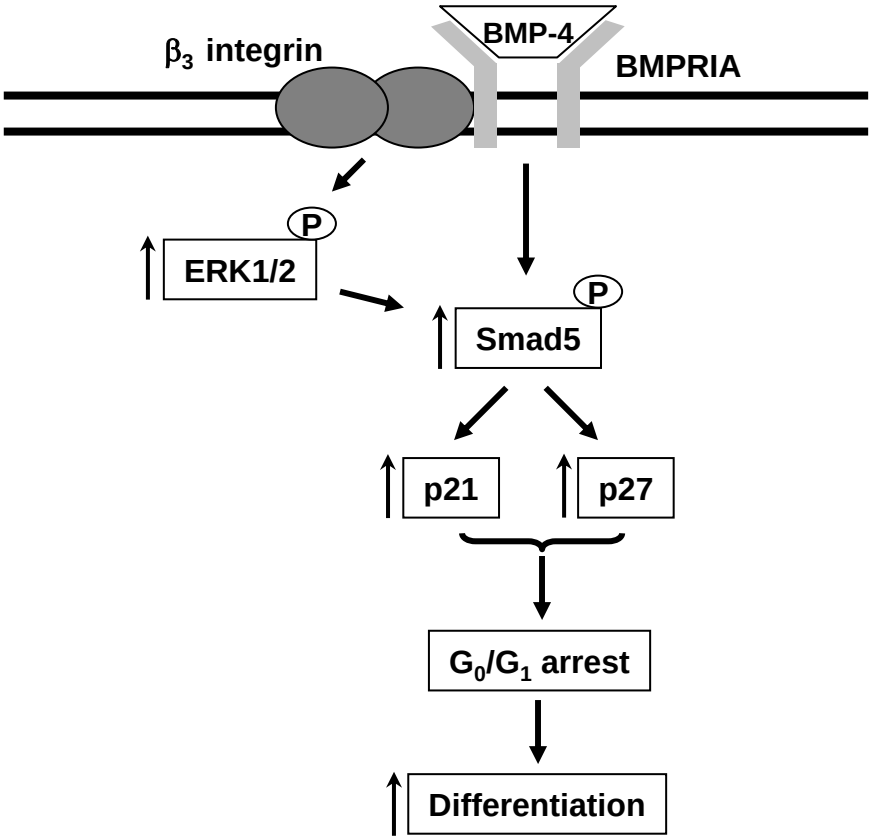



**Figure 5-1. Schematic representation of the signaling pathways regulating BMP-4-induced cell cycle arrest and differentiation in osteoblast-like cells.** BMP-4-induced $G_0/G_1$ cell cycle arrest and differentiation are mediated by $\beta_3$ integrin/ERK through their modulation of BMPRIA-specific Smad5 activation, which leads to increases in the expressions of Cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. ↑: up-regulation by BMP-4.

**Figure 5-2**

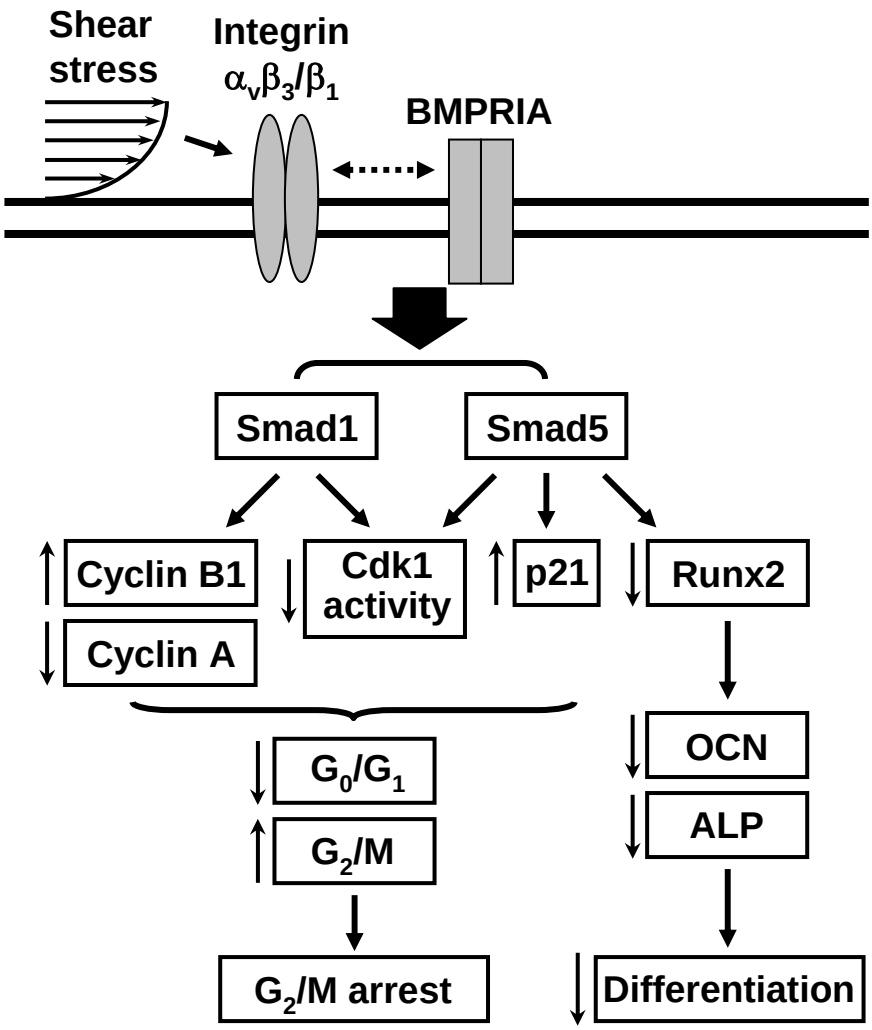



**Figure 5-2. Schematic representation of the signaling pathways regulating cell cycle and differentiation in tumor cells in response to shear stress.** ↑: up-regulation by shear. ↓: down-regulation by shear. Dotted double arrow line represents the interaction pathway that has not been defined.

# CHAPTER VI

# CONCLUSION

In the experiments studying the BMP signaling-mediated cell cycle distribution for regulating osteoblast functions under BMP-4 treatment, our study demonstrated that BMP-4 induces $G_0/G_1$ cell cycle arrest and hence cell differentiation in osteoblast-like cells. These BMP-4-induced responses are mediated by the $\beta_3$ integrin/ERK through their modulation of the BMPRIA-specific Smad5 activation, which leads to an increase in the expression of Cdk inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. Our findings provide a molecular basis for the mechanisms by which BMP-4 signaling induces the increase in the expression of $p21^{CIP1}$ and $p27^{KIP1}$, which play critical roles in the differentiation commitment process in osteoblasts.

In the experiments studying the BMPs signaling-mediated tumor cell growth under mechanical flow forces, our study demonstrated for the first time that shear stress induces a $G_2/M$ cell cycle arrest in tumor cells and inhibits cell differentiation. These shear-induced responses are mediated by the $\alpha_v\beta_3$ and $\beta_1$ integrins through their modulations of the BMPRIA-specific Smad activation, which leads to changes in expression and activity of the cell cycle regulatory proteins and inhibition of the Runx2 binding activity. The current study advances the new notion that mechanical forces are natural regulators of tumor biology. Our data on the shear-modulations of the cell cycle and differentiation in tumor cells suggest that the mechanical microenvironment of tumor cells and BMP signaling may play important roles in tumor development and pathology, and should be taken into account in tumor therapy and management.

# CHAPTER VII

# FUTURE WORKS

Accumulating data have demonstrated that BMP signaling impacts important molecular mechanisms in regulating not only bone cells but a variety of cell types, including tumor cells. Our data also demonstrated that BMP signaling may mediate the cell cycle distribution in order to regulate the BMP-4-induced osteoblast differentiation and mechanical flow force-induced tumor cell growth arrest.

There has been much exciting progress in understanding the BMP signaling applications in fracture healing and the tumorigenesis inhibition of tumor cells. In clinical settings, BMPs are used to treat open fractures, delayed union of fractures and spinal fusion [147-148]. BMPs have also been used to treat articular cartilage defects and degenerated intervertebral disks in animal experiments [149]. In those situations, BMPs may induce osteogenesis or chondrogenesis from mesenchymal cells. As the secrets of BMP signaling are progressively enlightened through scientific investigation, their roles in tumorigenesis grow in complexity. BMP signaling may function in both oncogenes and tumor suppressors, depending on the relative dosage and disease stage. While the canonical Smad signaling cascade has been elucidated, the specificities of the signals induced by the BMP-activated R-Smads in bone, cartilage and others cell types are not fully understood. Since BMPs transduce disparate responses in different cell types, it is important to understand the specificities of distinct receptor molecules, Smad proteins, and Smad regulators in different tissues. Moreover, the Smad-dependent pathway by itself does not explain how BMPs exert such diverse functions. It will be important to identify proteins that cooperate with Smads in signal transduction for different cell functions. The modulation of the activities of BMP signaling may be useful in establishing new approaches to the treatment of a variety of bone, cartilage, or tumor disorders.

The effect of the mechanical microenvironment in normal and tumor cells is another important mediator. Mechanical flow forces are most extensive study targets. The effects

of mechanical flow forces in tumor cells have been proposed for many years. However, the detailed molecular mechanisms are extremely unclear. Our data contribute novel information and may initiate new directions for investigating the mechanical flow force effects. The communication between mechanical flow forces and BMP signaling may play an important role in treating tumor patients, and further investigations are needed.

In this thesis, we used laminar shear stress at 12 dynes/cm$^2$ as a model to study the effect of mechanical forces on bone and tumor cell biology. However, the mechanical microenvironment around bone and tumor tissues is complex, which may include not only laminar, but also oscillatory types of flow, with different magnitudes of shear stress. Moreover, interstitial flow-induced shear stress on bone and tumor tissues is vary, with 8-30 dynes/cm$^2$ in bone and 0-12 dynes/cm$^2$ in tumor. Thus, our experimental model has some limitations in the study of the mechanical responses of bone or tumor cells. Nevertheless, our findings may provide insights into the molecular basis of mechanisms by which chemical and mechanical stimuli, such as BMPs and shear stress, regulate signaling, gene expression, and function in bone and tumor cells. However, it would be useful to design experimental models that can mimic more closely the physical microenvironments of bone and tumor cells for studying their responses to mechanical stimuli.

# CHAPTER VIII

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# CHAPTER IX

# NOTES ADDED IN PROOF

In the study of Bone Morphogenetic Protein-4-modulated Osteoblast $G_0/G_1$ Arrest and Differentiation, we further studied how the β3 integrin-ERK pathway regulates the BMP-4 effects. The detailed results and discussion will be elucidated below.

***9.1 BMP-4-induced ERK1/2 and Smad1/5 activations through the association of β3 integrin, FAK, and Shc in MG63.*** In the previously studies, we demonstrated that BMP-4-induced the ERK1/2 and Smad1/5 phosphorylation was mediated by β3 integrin. To elucidate how β3 integrin mediated the ERK1/2 and Smad1/5 activation in MG63, the cells were transfected with dominant-negative mutants of focal adhesion kinase (FAK) and Shc, and then kept as controls or stimulated with BMP-4 (25 ng/mL) for 30 min. The BMP-4-induced ERK1/2 and Smad1/5 phosphorylation were inhibited by transfection of MG63 cells with dominant-negative mutants of FAK and Shc (compared to empty vector pcDNA3) (Figure 9-1A). We further investigated the interaction between the integrin and FAK and Shc in response to BMP-4. MG63 cells were kept as controls or treated with BMP-4 (25 ng/mL) for 10 and 30 min, and their extracts were immunoprecipitated with an antibodies against β3 integrin. The BMP-4 stimulation resulted in a significant increase in the association of β3 integrin with FAK and Shc in 10 min, then decrease in 30 min (Figure 9-1B). These result suggesting that β3 integrin mediated BMP-4-induced ERK1/2 and Smad1/5 activation through the downstream signaling, FAK and Shc by an increase in the association of β3 integrin and FAK and Shc.

***9.2 BMP-4-induced Smad activations and hence p21$^{CIP1}$ and p27$^{KIP1}$ expression are mediated by ERK2, but not ERK1, in MG63.*** To investigate the exactly role of ERK1/2 in BMP-4-induced Smad1/5 activation, the cells were pre-treated with DMSO or MEK inhibitor, PD98059, and then kept as controls or stimulated with BMP-4 (25 ng/mL) for 30 min. The BMP-4-induced ERK1/2 and Smad1/5 phosphorylations were inhibited by pre-treating the PD98059 (Figure 9-2A). The cells were transfected with ERK1-specific siRNA (40 nM) and then kept as controls or stimulated with BMP-4 (25 ng/mL) for 30 min. When MG63 cells were transfected with ERK1-specific siRNA, which caused 80-90% reduction in the protein expression of ERK1, their BMP-4-induced Smad1/5 phosphorylation have no significant effects, compared to control siRNA (Figure 9-2B). We further elucidated if ERK1/2 mediated the BMP-4 induced the p21$^{CIP1}$ and p27$^{KIP1}$ expression. The cells were transfected with ERK1- and ERK2-specific siRNA (40 nM for each) and then kept as controls or stimulated with BMP-4 (25 ng/mL) for 48 h. The transfection of MG63 cells with ERK2-specific siRNA (compared with control siRNA) abolished the BMP-4-induced the p21$^{CIP1}$ and p27$^{KIP1}$ expression (Figure 9-2C). In contrast, ERK1-specific siRNA did not have inhibitory effects on the BMP-4-induced the p21$^{CIP1}$ and p27$^{KIP1}$ expression. These results suggest that the BMP-4 induced Smad1/5 activation and then p21$^{CIP1}$ and p27$^{KIP1}$ expression is mediated by ERK2, but not ERK1.

***9.3 BMP-4-induced $G_0/G_1$ arrest in MG63 cells is mediated by Smad1 and ERK2 activation***

***and p21$^{CIP1}$ and p27$^{KIP1}$ expression.*** In the previously studies, we demonstrated that BMP-4-induced the p27$^{KIP1}$ expression were mediated by Smad1 and BMP-4-activated ERK2 to mediate the p21$^{CIP1}$ and p27$^{KIP1}$ expression. To further elucidated the important of BMP-4-activated ERK2 and Smad1 and BMP-4-upregulated p21$^{CIP1}$ and p27$^{KIP1}$ expression in modulating the BMP-4-induced $G_0/G_1$ arrest, MG63 cells were transfected with ERK2-, p21$^{CIP1}$-, and p27$^{KIP1}$-specific siRNA (40 nM for each) and then kept as controls or treated with BMP-4 (25 ng/mL) for 72 h. For unstimulated cells, the transfection with ERK2-, Smad1-, p21$^{CIP1}$-, and p27$^{KIP1}$-specific siRNA (compared with control siRNA) did not alter their cell cycle distribution (Table 9-1). After BMP-4 treatment, the MG63 cells transfected with ERK2-, Smad1-, p21$^{CIP1}$-, and p27$^{KIP1}$-specific siRNA had a significantly lower cell percentage in the $G_0/G_1$ phase and a higher cell percentage in synthetic and $G_2$/M phases, as compared with cells transfected with the control siRNA (Table 9-1).

Our new data in this chapter clearly elucidates more details of the mechanisms between β3 integrin and ERK1/2 in the BMP-4 effects. FAK and Shc are both well known downstream molecules that are involved in the integrin-mediated cell functions. Our results show that β3 integrin mediated the ERK1/2 and Smad1/5 activation through the association with FAK and Shc. Moreover, our data also demonstrated that BMP-4-induced the ERK2 and Smad1 phosphorylation and p21$^{CIP1}$ and p27$^{KIP1}$ expression are all important in modulating the $G_0/G_1$ arrest in MG63 cells. These results more augment and extend the

understanding of the complex mechanisms that regulate the induction of osteoblast differentiation by BMP-4.

**Table 9-1. BMP4-induced $G_0/G_1$ arrest in MG63 cells is mediated by Smad1, ERK2, p21, and p27.**

| siRNA | Control (72 h) | | | BMP-4 (72 h) | | |
|---|---|---|---|---|---|---|
| | % of cells (mean ± SEM ) | | | % of cells (mean ± SEM ) | | |
| | $G_0/G_1$ | Synthetic | $G_2/M$ | $G_0/G_1$ | Synthetic | $G_2/M$ |
| **siCL** | 74.4±1.5% | 14.6±0.6% | 11.0±0.9% | 88.9±1.1%* | 5.6±0.5%* | 5.5±0.6%* |
| **siSmad1** | 69.5±1.1% | 14.8±0.5% | 15.7±0.6% | 74.2±3.0%*,# | 12.0±0.5%*,# | 13.8±2.5%# |
| **siERK2** | 73.0±1.3% | 10.5±0.3% | 16.5±1.6% | 71.2±3.3%# | 11.8±2.4%# | 17.0±0.8%# |
| **sip21** | 74.0±0.4% | 15.8±0.4% | 10.2±0.1% | 72.9±1.5%# | 16.6±1.2%# | 10.5±0.3%# |
| **sip27** | 70.0±0.8% | 18.0±2.4% | 12.0±1.6% | 74.9±1.9%*,# | 14.8±1.6%*,# | 10.3±0.3%# |

MG63 cells were transfected with control siRNA (siCL) or specific siRNA of Smad1 (siSmad1), ERK2 (siERK2), p21 (sip21) or p27 (sip27) (40 nM) for 48 h, and then were kept as controls or treated with BMP-4 (25 ng/ml) for 72 h. The cells were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in $G_0/G_1$, synthetic, or $G_2/M$ phases of the cell cycle. Data are mean ± SEM from three independent experiments. *, $P < 0.05$ *vs*. unstimulated control cells; #, $P < 0.05$ *vs.* cells transfected with control siRNA.

**Figure 9-1**

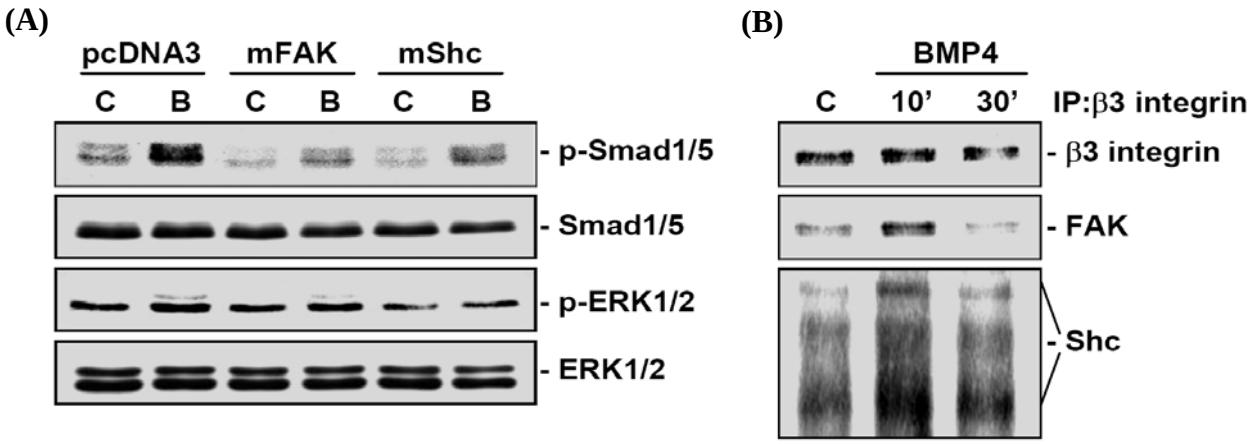


**Figure 9-1. BMP-4-induced ERK1/2 and Smad1/5 activations through the association of β3 integrin, FAK, and Shc in MG63 cells.** (*A*) MG63 cells were transfected with 3 μg of Shc-SH2 (mShc), FAK(F397Y) (mFAK), or pcDNA3 empty vector for 48 h prior to treatment with BMP-4 (25ng/mL) (B) for 30 min. (*B*) MG63 cells were kept as controls (C) or treated with BMP-4 (B; 25 ng/ml) for 10 and 30 min. The associations of β3 integrin with FAK and Shc were determined by immunoprecipitation assay and Western blot analysis, as described in Materials and Methods. Results are representative of triplicate experiments with similar results.

**Figure 9-2**

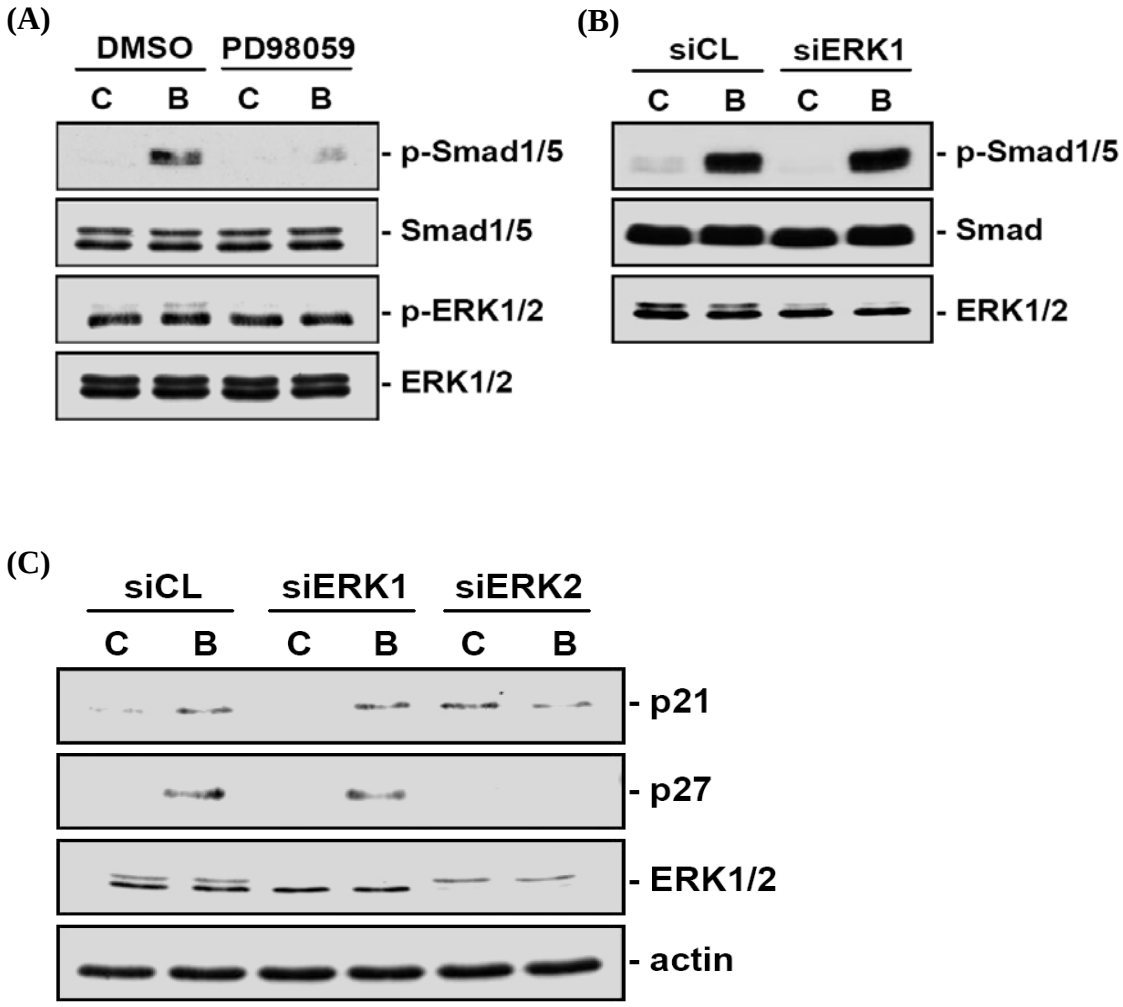


**Figure 9-2. BMP-4-induced Smad1/5 activations and hence $p21^{CIP1}$ and $p27^{KIP1}$ expression are mediated by ERK2 in MG63 cells.** (*A*) MG63 cells were pre-treated with DMSO or PD98059 (30 μg/mL), for 1 h, and kept under static conditions (C) or treated with BMP-4 (25ng/mL) (B) for 30 min. (*B*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of ERK1 (siERK1) (40 nM each) for 48 h prior to treatment with BMP-4. (*C*) MG63 cells were transfected with control siRNA (siCL) or specific siRNA of ERK1 (siERK1) and ERK2 (siERK2) (40 nM each) for 48 h prior to treatment with BMP-4. Results are representative of triplicate experiments with similar results.

# CHAPTER X

# PUBLICATIONS

1. **Chang SF**, Chang CA, Lee DY, Lee PL, Yeh YM, Yeh CR, Cheng CK, Chien S, Chiu JJ. 2008. Tumor cell cycle arrest induced by shear stress: Roles of integrins and Smad. ***Proc Natl Acad Sci U S A.*** 105:3927-3932.
2. **Chang SF**, Chang CA, Chang TK, Peng HH, Yeh YT, Lee DY, Yeh CR, Cheng CK, Chiu JJ. 2008. Bone Morphogenetic Protein-4 Induces G0/G1 Arrest and Differentiation in Osteoblast-like Cells *via* Increased Expressions of $p21^{CIP1}$ and $p27^{KIP1}$: Roles of integrins, extracellular signal-regulated kinase, and Smad. ***J Bone Miner Res.***
3. Lee DY, Yeh CR, **Chang SF**, Lee PL, Chien S, Cheng CK, Chiu JJ. 2008. Integrin-mediated expression of bone formation-related genes in osteoblast-like cells in response to fluid shear stress: roles of extracellular matrix, Shc, and mitogen-activated protein kinase. ***J Bone Miner Res.*** 23:1140-1149.
4. Chen CN, **Chang SF**, Lee PL, Chang K, Chen LJ, Usami S, Chien S, Chiu JJ. 2006. Neutrophils, lymphocytes, and monocytes exhibit diverse behaviors in transendothelial and subendothelial migrations under coculture with smooth muscle cells in disturbed flow. ***Blood.*** 107:1933-1942.
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6. Chiu JJ, Chen LJ, **Chang SF**, Lee PL, Lee CI, Tsai MC, Lee DY, Hsieh HP, Usami S, Chien S. 2005. Shear stress inhibits smooth muscle cell-induced inflammatory gene expression in endothelial cells: role of NF-kappaB. ***Arterioscler Thromb Vasc Biol.*** 25:963-969.
7. Chiu JJ, Lee PL, Chen CN, Lee CI, **Chang SF**, Chen LJ, Lien SC, Ko YC, Usami S, Chien S. 2004. Shear stress increases ICAM-1 and decreases VCAM-1 and E-selectin

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**Awards:**

2008 年交通大學生科院生物科技學術研討會優秀壁報論文獎

2008 年第十六屆細胞與分子生物新知研討會優秀壁報論文獎

2004 年第七屆工程科技與中西醫藥應用研討會學生論文獎第一名

**Conference Poster:**

2008 年交通大學生科院生物科技學術研討會

- Tumor cell cycle arrest induced by shear stress: Roles of integrins and Smad
- $p21^{CIP1}$ and $p27^{KIP1}$ are associated with Bone Morphogenetic Protein 4-induced MG63 osteoblast-like cell growth arrest and differentiation

2008 Experimental Biology Conference

- Tumor cell cycle arrest induced by shear stress: Roles of integrins and Smad

2008 年第十六屆細胞與分子生物新知研討會

- Tumor cell cycle arrest induced by shear stress: Roles of integrins and Smad

2004 年第七屆工程科技與中西醫藥應用研討會

- Analysis of the effect of disturbed flow on leukocyte transendothelial migration in a co-culture model of the arterial wall.