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BIOPHYSICAL REGULATION OF HUMAN MESENCHYMAL STEM CELL PROLIFERATION

A Thesis in

Cellular and Molecular Biology

by

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ABSTRACT

Osteoporosis, a disease characterized by low bone mass and increased fracture susceptibility, affects more than 10 million Americans and an additional 34 million are at risk of developing this disease (1). As our population continues to age these numbers are likely to grow significantly. Estimates of the economic burden of treating this disease in 2005 are on the order of $17 billion and are expected to increase by as much as 50% by 2025 (2). As a result, the development of new therapeutic techniques designed to enhance bone formation and strength is a necessity. One way to achieve such goals is to harness the proliferation and differentiation potential of mesenchymal stem cells (MSCs) derived from skeletal tissue. MSCs provide a source of bone-forming osteoblasts important for skeletal homeostasis, but the factors that regulate the proliferation and differentiation of MSCs are not completely understood.

Mechanical signals are widely accepted as regulators of skeletal homeostasis, such that the addition of exogenous mechanical load enhances osteoblastic bone formation while inhibiting osteoclastic bone resorption (3). Current hypotheses suggest that the deformation of bone in response to an applied load generates substrate strains that drive the movement of interstitial fluid and bone cells sense this fluid movement via changes in chemotransport or shear stress across cell bodies and processes (4-6). Indeed, numerous *in vitro* studies suggest that both osteoblastic and osteocytic cells respond to the predicted physiologic levels of fluid shear stress by releasing paracrine factors necessary for the anabolic response of bone to mechanical loads (7-9). It is likely that fluid flow also regulates the behavior of MSCs, but there is little experimental evidence for this and the signaling cascades activated in MSCs in response to fluid flow have not been examined.

Thus the goals of this thesis are three-fold: 1) to examine the effect of oscillatory fluid flow on human mesenchymal stem cell (hMSC) proliferation and to identify candidate signaling cascades involved in this response; 2) to identify the factor that initiates the activation of these signaling cascades; and, 3) to identify the biophysical signal that hMSCs perceive.

One hour of fluid flow exposure induced a significant increase in cellular proliferation over static controls, indicating that like osteoblasts and osteocytes, hMSCs are responsive to fluid flow. Since intracellular calcium is a vital mediator of the processes by which extracellular signals are conveyed to the cell's interior, we next examined whether calcium signaling pathways contribute to the effect of fluid flow on hMSC proliferation. Fluid flow exposure triggered a robust, but transient increase in intracellular calcium concentration that was partially mediated by the activation of phospholipase C. Increases in intracellular calcium concentration stimulated the activation of calcineurin and the nuclear translocation of its target transcription factor, nuclear factor of activated T cells (NFAT). Fluid flow also stimulated the phosphorylation of the MAP kinases, ERK-1 and -2. Pharmacological inhibition of calcineurin activation or ERK1/2 phosphorylation blocked the effect of fluid flow on hMSC proliferation.

Having determined that fluid flow stimulates hMSC proliferation, we attempted to identify the factor(s) responsible for this response. As previous studies from our laboratory and others suggested a role for extracellular ATP in osteoblastic and osteocytic mechanotransduction (7,10,11), we hypothesized that the release of ATP may also account for the mechanosensitivity of hMSCs. hMSCs actively release ATP in response

to fluid flow stimulation and express a number of purinergic receptors necessary to respond to extracellular nucleotides. Treating hMSCs with ATP, but not other nucleotides, induced cellular proliferation at a level similar to that observed with fluid flow. Further, enzymatically degrading extracellular nucleotides with apyrase abolished the effect of fluid flow on hMSC proliferation. Degrading extracellular nucleotides also abrogated the effect of fluid flow on intracellular calcium signaling, the activation of calcineurin, and the nuclear translocation of NFAT. These data strongly suggest that ATP is the factor that mediates the induction of hMSC proliferation in response to fluid flow.

Finally, we examined the contribution of chemotransport and fluid shear stress, two biophysical signals induced by interstitial fluid flow, to the effect of fluid flow on hMSC proliferation. Alterations in chemotransport, but not fluid shear stress, affected the sensitivity of hMSCs to fluid flow. We found that decreasing chemotransport inhibited hMSC proliferation as well as intracellular calcium signaling and ATP release in response to fluid flow. In contrast incrementally increasing fluid shear stress did not alter any of these parameters. These data suggest the clearance of cellular metabolites and replacement of nutrient levels are a prerequisite for hMSC mechanotransduction.

In summary, these studies provide new evidence that mechanical signals regulate the behavior of mesenchymal stem cells and outline for the first time the molecular mechanisms by which fluid flow affects these cells. The similarities between the signaling cascades activated by fluid flow in more mature osteoblastic cells and in hMSCs imply that a common pathway exists by which mechanical signals are translated to cellular responses. These data could be used in the development of new therapeutic

techniques designed to enhance the recruitment of mesenchymal stem cells and promote their proliferation and subsequent differentiation into bone-forming osteoblasts. Such techniques would be beneficial in the treatment of diseases in which decreased bone formation compromises the integrity of skeletal tissue. Additionally, given the interest in hMSCs in tissue engineering protocols, these data could be applied in such a way as to control the phenotype of stem cells *in vitro* and drive their proliferation and differentiation towards specific lineages.

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CHAPTER 1

BONE STRUCTURE AND COMPOSITION

The 206 bones of the human skeleton are composed of a specialized form of connective tissue that performs at least five vital functions. The rigidity of the skeleton provides the scaffolding that gives the human body its shape, while its strength serves to protect vital organs. This is exemplified by the encasement of the brain within the skull and encirclement of the heart and lungs by the rib cage. Through the attachment of muscles, ligaments and tendons and the flexibility of joints, bones provide a lever system that allows locomotion as well as respiration. The deposition of hydroxyapatite in bone's extracellular matrix serves as a readily accessible source of calcium and phosphate, as well as many other minerals. Additionally, bone serves as a site of post-natal hematopoiesis as bone marrow fills the cavities of long bones.

Despite these important functions, bone is a dynamic tissue with old or damaged bone being resorbed and replaced by new bone matrix. Normally, the rate of bone formation equals that of degradation and net bone mass is maintained, but pathological conditions develop if this balance becomes dysregulated. If osteoblastic bone production is decreased or osteoclastic bone resorption is increased, bone mass is lost and may lead to the development of osteopenia and osteoporosis with an increase in fracture risk. If osteoblastic bone production is increased or osteoclastic bone resorption decreased, bone mass is gained and may lead to the development of osteopetrosis that potentially impairs hematopoiesis. A number of factors, including biophysical signal and hormonal agents, have been identified that regulate the balance between bone formation and bone degradation.

1.1 Bone Structure

Based upon their structure and mode of development, bones can be classified in two broad groups: long bones like the femur, radius, and ulna, and flat bones like the skull, mandible, and scapula. Long bones, generally found in the limbs where they are utilized in locomotion and carry associated loads, are composed of a long, marrow-filled tube called the diaphysis. The flared ends of the diaphysis, called the metaphysis, connect it to the epiphysis that caps each end. In pre-pubertal mammals, the metaphysis is separated from the epiphyis by the epiphyseal plate or growth plate. A thin layer of cartilage in this region allows for the longitudinal growth of these bones. Following puberty, the metaphysis and epiphysis fuse and the epiphyseal plate is replaced by bone. Flat bones, which function primarily in the protection of vital organs, lack marrow spaces and are thin in one dimension and longer in the other two (12,13). Figure 1-1 illustrates the structure of a long bone.

The diaphysis of long bones as well as the outer shell of flat bones is composed mainly of cortical bone. This type of bone tissue is a dense, solid mass with only a small fraction of empty space in the forms of Haversian systems and Volkmann's canals that contain blood vessels necessary to supply the tissue's interior. The epiphysis, metaphysis, and interior of flat bones are composed of cancellous bone. In contrast to the dense structure of cortical bone, cancellous bone has a honeycomb-like shape composed of plates and rods called trabeculae. The arrangement of trabeculae is highly variable and the spaces not filled with bone matrix contain marrow (13-15). Whereas the main function of cortical bone is to provide mechanical support, the primary function of cancellous bone is metabolic (16).

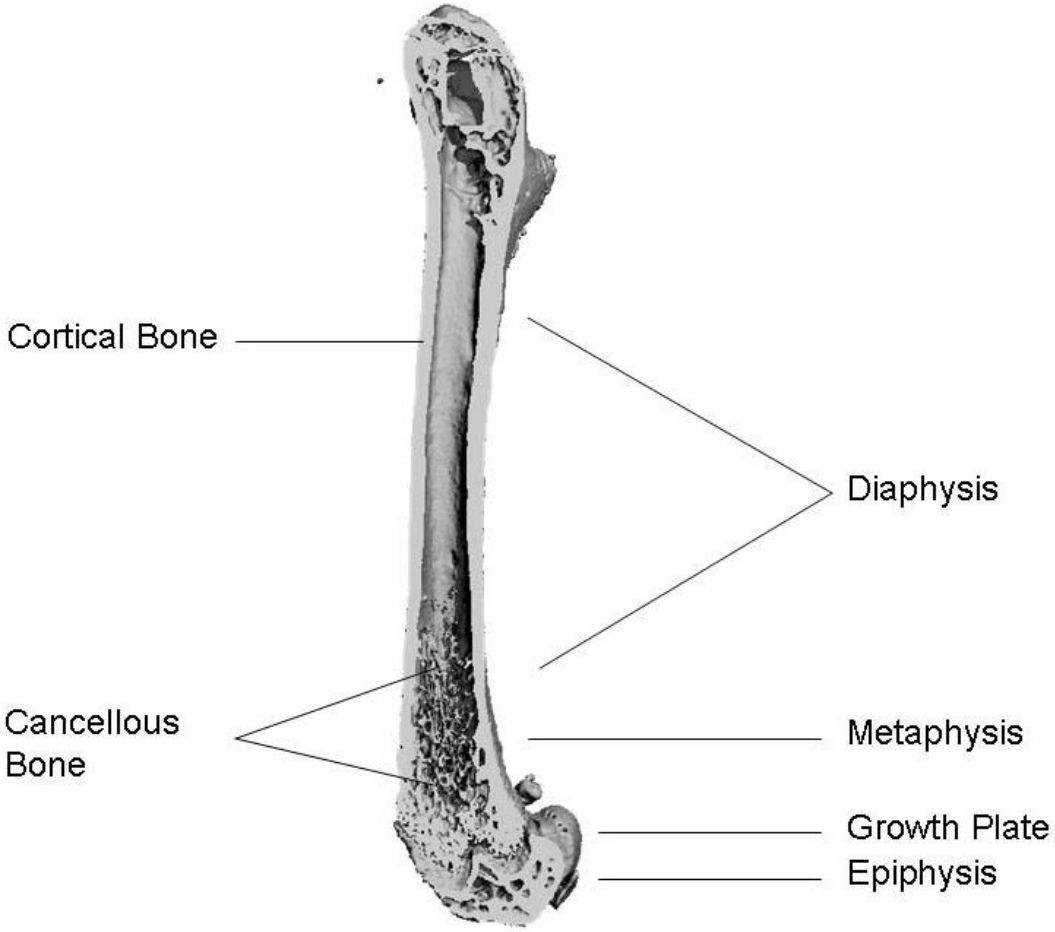

Figure 1-1 Example of long bone structure
Computer rendering of microCT images of a mouse femur.

Cortical and cancellous bone tissue can be further divided into woven or lamellar bone. During embryonic development, periods of rapid growth and fracture healing, bone matrix is rapidly deposited in a disorganized manner known as woven bone. In this type of bone tissue, collagen fibers are interwoven and bone cells are randomly distributed throughout the matrix. Eventually, woven bone is replaced by a more highly organized and structurally sound tissue known as lamellar bone. Lamellar bone is deposited in discreet layers with a thickness of approximately 3-7μm called lamellae. Within each lamella the collagen fibers are deposited in a highly organized and parallel fashion, but the fiber direction within adjacent lamellae may differ significantly. In cortical bone three patterns of lamellae exist. First, the circumference of long bones is surrounded by several uninterrupted layers of lamellae known as circumferential lamellae. Second, in the interior, concentric lamellae contain circular rings of lamella that surround a central vascular canal. Third, interstitial lamellae fill the spaces between concentric lamellae and represent fragments of resorbed concentric or circumferential lamellae (13-15)

All bone surfaces are covered by a layer of fibrous connective tissue. The periosteum covers all of the outer surfaces of bones with the exception of joints which are lined with a layer of articular cartilage. The endosteum covers inner bone surfaces including all trabecular surfaces and the walls of Haversian and Volkmann canals. Both of these layers are perforated by blood vessels that supply bone tissue and nerve endings, and contain the cells necessary for bone formation and resorption (12-14).

1.2 Bone Composition

Mature bone matrix contains both a mineral phase and an organic phase with a typical volumetric ratio of 65% mineral to 35% organic matrix (13). The mineral phase consists primarily of hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, that in addition to providing bone with its strength and rigidity serves as a readily available store of calcium and phosphate. Impurities, such as carbonate, fluoride, and citrate, are also sometimes incorporated in this phase (13,14). The organic phase is composed of extracellular matrix proteins that provide bone with its flexibility.

Type I collagen represents approximately 90% of bone's organic phase. Each molecule consists of a helical trimer of two α1 chains and 1 α2 chain. Osteoblasts (discussed in the next section) secrete type I collagen as a propeptide that requires cleavage of C- and N-telopeptides. Proteolytic processing allows mature molecules to assemble in an overlapping pattern into fibrils. The space between each molecule is thought to serve as a nucleation site for mineralization (17,18). Bone's tensile strength is derived from type I collagen (14). The importance of this molecule within skeletal tissue is clearly evident in Osteogenesis Imperfecta. Genetic mutations in the genes encoding either the α1 chains or α2 chain lead to severe fragility of bone (19,20).

The remaining 10% of bone's organic phase is composed of proteoglycans and other non-collagenous matrix proteins. Examples include osteocalcin, osteopontin, osteonectin, decorin, and biglycan. The functions of these molecules include, but are not limited to: assisting in collagen fiber assembly, nucleating and controlling the rate of mineralization, mediating cell attachment, acting as chemoattractants, and binding growth factors (18,21-23).

1.3 Bone Cell Types

Maintenance of bone mass is controlled by several cell types derived from two separate lineages and include mesenchymal osteoblasts, osteocytes, bone lining cells, and hematopoietic osteoclasts.

1.3.1 Osteoblasts

The osteoblast is responsible for the synthesis, deposition, and mineralization of bone matrix. Derived from mesenchymal stem cells (discussed in Chapter 3) present in the bone marrow, three maturational stages have been identified in osteoblast development (24,25). During the proliferative phase, osteoprogenitors/preosteoblasts undergo a series of population doublings prior to acquiring a cuboidal shape at the bone surface (26). Cells in the proliferative phase exhibit elevated expression levels of the AP-1 transcription factors c-fos and c-jun (27) and cell cycle related proteins (28,29). As cells lose their proliferation capacity and enter the matrix maturation phase, the levels of cyclin B and E are increased (30). Additionally, expression levels of bone matrix proteins including type I collagen, osteopontin, and bone sialoprotein peak (31-33) and are accompanied by the expression of alkaline phosphatase and the PTH/PTHrP receptor (34,35). Morphologically, osteoblasts in this phase have enlarged Golgi complexes and an extensive endoplasmic reticulum necessary for matrix secretion (36). In the final phase of osteoblast development, osteoblasts proceed to mineralize the matrix they produce. Osteoblasts release vesicles containing calcium and phosphate ions necessary for the mineralization of bone as well as nucleating enzymes such as alkaline phosphatase, adenosine triphosphatase, and pyrophosphatase (18,37).

Having completed the maturation process, most osteoblasts will die via apoptosis (38,39). A small fraction will acquire one of two "terminally" differentiated phenotypes, that of an osteocyte or a bone-lining cell (Figure 1-2)

1.3.2 Osteocytes

If an osteoblast becomes encapsulated within the matrix it produces, it acquires a stellate morphology (40), and is referred to as an osteocyte. Human bone contains approximately 10 times as many osteocytes as osteoblasts, making this the most abundant bone cell type (41). The osteocyte cell body resides within a small cavity called a lacunae and thin cellular processes permeate the bone matrix in channels called canaliculi. While few osteocyte-specific markers have been identified, osteocytes exhibit high levels of osteocalcin, osteopontin, and dentin matrix protein-1 expression, while only weakly expressing alkaline phosphatase (42-44). The expression of these phenotypic markers of osteoblastic cells as compared to others may enable osteocytes to inhibit the mineralization of the lacunar-canalicular system (45).

Functionally, osteocytes are thought to communicate changes within the skeletal tissue, such as mechanical loads, to effector cells on the bone surface (46,47). The uniform distribution of osteocytes throughout the bone matrix is ideal for such a function. Further, dendritic processes allow communication with neighboring osteocytes as well as cells on the bone surface and within the marrow space via the formation of functional gap junctions (48-50). Indeed, Taylor *et al*. reported that osteocytes are uniquely able to communicate mechanical signals to osteoblastic cells and increase their activity (51). Conversely, osteocyte apoptosis is associated with increased bone turnover by osteoclasts (52), and this may be one way that microdamage is detected within the bone matrix

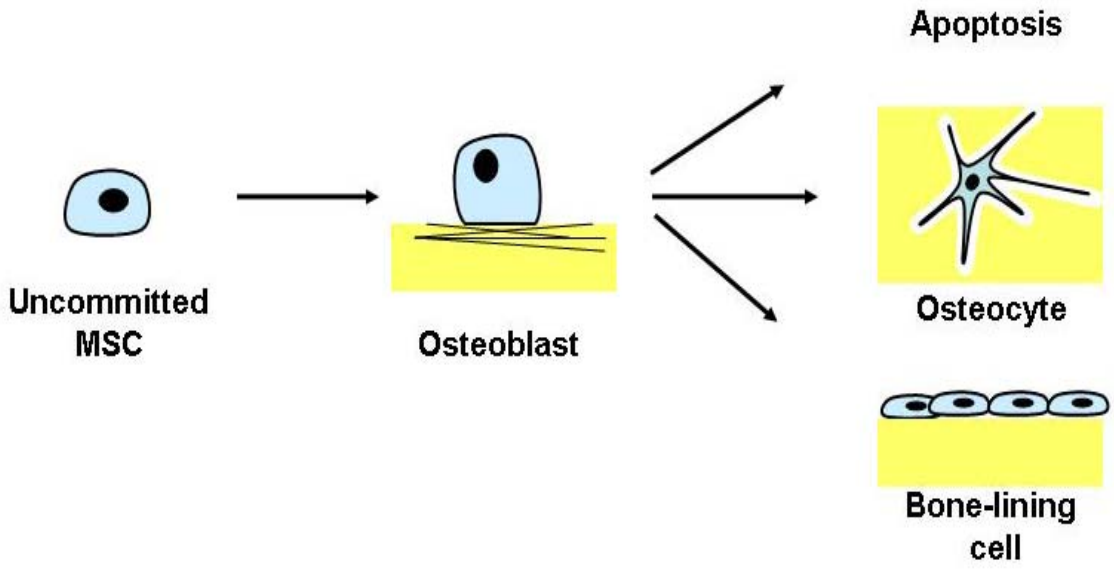

Figure 1-2 The osteoblast lineage

(53,54). Recent evidence suggests that osteocytes may also play a role in regulating mineral metabolism (55) as well as matrix properties via remodeling of the lacunae (56).

1.3.3 Bone lining cells

Bone lining cells represent the second "terminally" differentiated phenotype of osteoblastic cells. These thin, polygonal shaped cells cover all periosteal and endosteal bone surfaces (40) as well as trabecular surfaces (57). In general, these cells are not thought to be involved in the bone formation processes (58). Indeed, morphological studies reveal that this cell population has few cytoplasmic organelles (59,60). It is possible that lining cells constitute a membrane separating bone fluids from interstitial fluid and help to maintain the ionic concentrations necessary for mineralization (58,61,62). There is also some evidence to suggest that bone lining cells are capable of reverting to their osteoblastic phenotype. Mechanical strain and PTH treatment are followed by rapid increases in bone lining cell metabolism (63-65). As discussed later, lining cells also likely play a role in preparing the bone surface for resorption (66).

1.3.4 Osteoclasts

Resorption of bone tissue is carried out by osteoclasts that are derived from hematopoietic cells (67,68). In response to osteoclastogenic signals, namely RANKL (discussed later) (69) and macrophage-colony stimulating factor (70), progenitor cells of the monocyte/macrophage lineage fuse to form multinucleated osteoclasts (71).

The effective degradation of bone requires the formation of a specialized compartment between the ruffled membrane of an osteoclast and the bone matrix. As osteoclastic precursors differentiate, they acquire the ability to tightly adhere to the bone matrix via $\alpha_v\beta_3$ integrins (72). Loss of β_3 integrin expression or function impairs

osteoclastic bone resorption and leads to a progressive increase in bone mass (73-75). To degrade the bone's mineral phase, a H^+-ATPase (76) and the ClC-7 chloride channel (77,78) are localized to the ruffled membrane. The actions of these two enzymes lead to the acidification of the resorption compartment (pH 4.5) and the mobilization of the mineral phase (79,80). Secretion of acid proteases like cathepsin K, MMP9, and MMP13 leads to the degradation of the organic component (81,82).

1.4 Mechanisms of Bone Development

The cells that compose the skeleton are derived from three developmental lineages (83,84). Neural crest cells give rise to the craniofacial skeleton. The axial skeleton is derived from paraxial mesoderm, while the limbs are derived from lateral plate mesoderm. Once cells from these lineages migrate to sites of future skeletal tissue, bone formation occurs via one of two mechanisms.

Flat bones like the clavicle and craniofacial bones develop via a process known as intramembraneous bone formation. Here, mesenchymal cells proliferate and form condensations prefiguring bony elements. Post-proliferative cells differentiate directly to osteoblasts that secrete the osteoid matrix (85).

Long bones of the axial skeleton and limbs develop via a more complicated process known as endochondral bone formation (83,85,86). Similar to intramembraneous bone formation, cells proliferate rapidly and form mesenchymal condensations. Rather than differentiate to osteoblasts, these cells differentiate to chondroblasts that secrete a matrix rich in type II collagen. Having produced a cartilaginous model of the future bone, cells at the midshaft increase in size, become hypertrophic, secrete type X collagen, and contribute to cartilage mineralization before undergoing apoptosis. Simultaneously,

perichondrial cells secrete a thin layer of bone that surrounds the midshaft and forms the bone collar. Invading blood vessels then deliver hematopoietic stem cells, osteoclasts, and osteoblasts. The calcified cartilage is removed and differentiated osteoblasts replace it with bone to form the primary ossification center. With continued development, the ossification center expands towards each end of the bone, while secondary ossification centers appear in the epiphyseal cartilage. Eventually the cartilaginous model is completely replaced by bone matrix with the exception of the growth plate that allows for longitudinal growth mediated by chondrocyte proliferation.

1.5 Bone Modeling and Remodeling

Alterations in skeletal architecture occur via one of two processes: bone modeling or remodeling. Differences between these two processes are outlined in Table 1-1.

Bone modeling changes the overall size and shape of individual bones through the independent deposition of bone matrix by osteoblasts or resorption by osteoclasts. During longitudinal bone growth, bone modeling is used to shape and reshape the diaphysis, epiphysis, and metaphysis. Further, the addition or removal of bone tissue at the periosteal or endosteal surface enables the tissue to achieve the optimum shape with regards to the mechanical demands placed upon it (14).

The bone remodeling process has been thoroughly outlined by Parfitt (66,87-91) and unlike bone modeling does not lead to a net change in bone shape. Rather, this process involves the removal of pre-existing and often damaged bone followed by its replacement with newly deposited bone. Requiring the activity of osteoclasts as well as osteoblasts at a given location, bone remodeling is cyclical in nature. In the first stage, a quiescent bone surface becomes “activated”. The selected surface may represent the wall of a

Table 1-1. Differences between modeling and remodeling

	Modeling	Remodeling
Cell types	Osteoblasts or Osteoclasts	Osteoblasts and Osteoclasts
Net change in shape	Yes	No
Timing	Continuous	Cyclical
Surfaces of resorption and formation	Different	Same
Change with age	Yes	No

After Parfitt (66)

Haversian canal, in the case of intracortical remodeling; the periosteal or endosteal surface of cortical bone; or, the surface of a trabecular structure and may be selected based upon its proximity to some form of microdamage (92). Bone lining cells release proteases necessary to degrade the endosteal/periosteal membrane (93) and then retract so that osteoclastic precursors can access the mineralized matrix. In the second stage, called "resorption", multinucleated osteoclasts resorb the old or damaged bone. When osteoclastic activity is completed, osteoblastic precursors are recruited to the site and begin to proliferate in response to mitogenic factors released from the degraded bone matrix (94,95) called "reversal". In the final stage, "formation", the osteoblasts replace the degraded bone matrix and return it to its quiescent state. Ideally, osteoblasts would deposit the same amount of bone that osteoclasts degrade, but this is rarely the case. Interestingly, while around 10 osteoclastic cells can remove the existing bone (96), several thousand osteoblastic cells are required to replace it (97). Figure 1-3 illustrates the 4 stages of bone remodeling.

1.6 Regulators of Bone Homeostasis

Skeletal homeostasis is regulated by a diverse list of factors, including mechanical signals, hormones, and autocrine/paracrine factors. Since the effects of mechanical signals on skeletal homeostasis are a focus of this thesis, these data are discussed in Chapter 2. Subsets of the systemic and local factors that regulate bone deposition and bone resorption are described below.

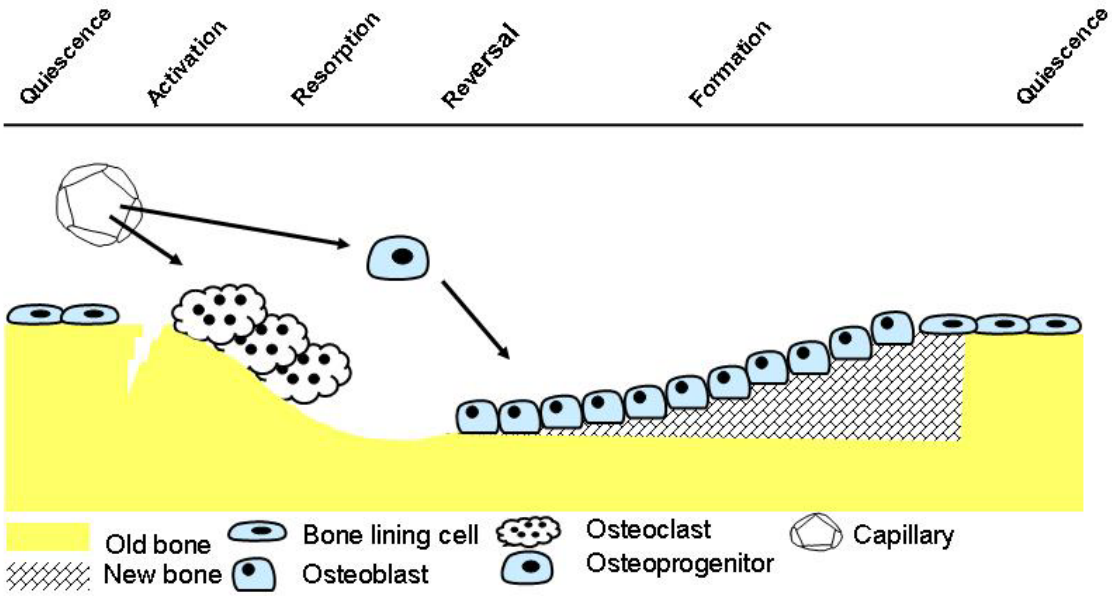

Figure 1-3 Bone remodeling sequence.
After Parfitt 1994 (89)

1.6.1 Hormonal regulators

Parathyroid hormone (PTH) is a major regulator of serum calcium levels by action on the kidney, where PTH enhances calcium absorption, and bone. As serum calcium levels decrease, PTH is released from secretory granules in parathyroid cells. When serum calcium levels begin to rise, PTH secretion is diminished. In bone, PTH stimulates bone resorption through actions on osteoblastic cells (98,99). In response to chronic PTH stimulation, osteoblastic cells exhibit increased expression of matrix metalloproteinases that degrade endosteal/periosteal membranes as in the remodeling process (100,101). Additionally, PTH induces a transient increase in osteoblast apoptosis (102), and enhances RANKL expression, while decreasing OPG expression (103,104) to increase osteoclast differentiation. Interestingly, intermittent exposure to PTH has an anabolic effect on bone mass. This effect appears to be mediated by a decrease in osteoblast apoptosis (105,106), the differentiation of osteoprogenitor cells (107), increased osteoblast number (108,109), and the activation of bone lining cells (63).

$1,25(OH)_2$ vitamin D_3 (Vitamin D_3) also affects bone mass by regulating calcium and phosphorus homeostasis. This steroid hormone is produced by the skin or metabolized by the liver and kidney, and enhances the absorption of calcium by the kidney and the intestine (110-112). Mice lacking the vitamin D receptor exhibit decreased serum calcium levels and low bone density compared to control mice (113,114). When dietary calcium intake is insufficient to maintain normal serum calcium levels, vitamin D_3 stimulates osteoclastic differentiation through the action of osteoblastic cells (115) and the increased expression of RANKL (116,117). *In vitro* studies indicate that vitamin D_3

inhibits PTH expression and secretion (118-120), suggesting a relationship between these two calciotropic hormones.

The effects of reproductive hormones on skeletal homeostasis are well established (121). Estrogen deficiency in postmenopausal women is associated with increased bone resorption (122,123), and is mirrored in animal models following ovariectomy (124,125). Estrogen replacement therapy depresses bone resorption in both humans (126,127) and animal models (128). On the cellular level, estrogen inhibits the development of osteoclastic precursors (129,130) and promotes osteoclast apoptosis (131), but the direct effects on osteoblasts may depend upon the stage of differentiation (132-134). Similarly, hypogonadism is associated with decreased bone mineral density in men (135). Androgen receptors are expressed by osteoblastic cells (136), and testosterone has been shown to enhance osteoblastic cell proliferation and differentiation (137,138).

1.6.2 Local regulators

The Osteoprotegerin/RANKL/RANK axis regulates the development and activity of osteoclasts (67). Osteoprotegerin (OPG) is a secreted tumor necrosis factor receptor family member that circulates *in vivo* (139) and inhibits osteoclastogenesis (139-141). Mice lacking this protein exhibit an osteoporotic phenotype from birth that is related to enhanced osteoclast formation and function (142,143). Later, it was revealed that OPG acts a decoy receptor that antagonizes the interaction of RANK and RANKL. The activation of RANK on the surface of osteoclastic precursors induces differentiation (69). The ligand for this protein is RANKL and is expressed by osteoblasts and marrow stromal cells (144-146). Genetic deletion of RANK or RANKL induces severe osteopetrosis and a lack of osteoclasts (147,148).

Members of the transforming growth factor-β superfamily regulate both the deposition of bone matrix by osteoblasts and its degradation by osteoclasts (149). TGF-β1, the most abundant TGF-β in bone (150), regulates all stages of osteoblast differentiation. Treating osteoprogenitor cells with TGF-β1 enhances cellular proliferation (151,152) and chemotaxis (153-155). During the early stages of matrix deposition, TGF-β1 enhances the expression of many extracellular matrix proteins including type I collagen, osteonectin, and osteopontin (151,156-158). However, the mineralization of bone matrix is inhibited by TGF-β1 as is osteocalcin expression (159-161). This effect may be involved in the prevention of osteoblast apoptosis and terminal differentiation into osteocytes (39,162). High concentrations of TGF-β1 have also been shown to inhibit osteoclast formation (163-165). These results are likely to be mediated by a decrease in osteoblastic RANKL expression and enhanced OPG expression (166,167).

Prostaglandins are released from bone cells in response to a number of stimuli including growth factors (168), cytokines (169), and exposure to mechanical signals (8,170). Initially, these factors were thought to have catabolic effects on bone mass (171,172). Indeed, treating bone marrow cultures with PGE_2 stimulates osteoclast formation (173,174), likely through an increase in the expression of RANKL (175,176). Further, knocking out expression of prostaglandin receptors impairs osteoclast formation (177-179). Later, Jee and colleagues found that prostaglandins also stimulate bone formation (180-184). This effect may be partially explained by an increase in the recruitment of osteoblastic progenitors from the bone marrow (185,186).

CHAPTER 2

BONE CELL MECHANOTRANSDUCTION

In order for bone to serve as an effective weigh-bearing structure, it must be able to withstand the functional loads placed upon it. As these loads change with activity levels and throughout a lifetime, skeletal tissue must possess the capacity to adapt to its mechanical environment. Commonly referred to as Wolff's Law (14,187), the adaptation of skeletal mass and architecture to changes in mechanical loads is well documented.

In the microgravity of space, the physical forces regulating skeletal homeostasis are greatly reduced and bone loss ensues. Astronauts on the Skylab missions lost as much as 4% of their bone mass through the course of their flight (188). Similarly, rats exhibited a decrease in bone mass resulting from a reduction in osteoblast number (189,190) and decreased rate of cortical and trabecular bone formation following spaceflight (191,192). Conversely, the addition of exogenous loads stimulates an increase in osteogenic activity and bone formation (3,193). Indeed, the sensitivity of bone cells to changes in their biophysical environment is such that only a single, short exposure to a dynamic load is necessary to activate bone-forming osteoblasts and increase bone formation (64). But, the effects of mechanical loads on skeletal homeostasis are dependent upon their magnitude. In humans, high impact activities are more osteogenic than low impact activities (194,195).

2.1 Frost's Mechanostat Theory and Mechanical Usage Windows

To explain the differential effects of mechanical loads, Frost likened bone's adaptive response to that of a thermostat or "mechanostat" with mechanical strain (a unit of deformation such that a 1% change in an object's length is equivalent to 10,000microstrain (μE)) being the key parameter (196,197). To exert an effect on skeletal mass, Frost hypothesized that strains must surpass a set point, which he labeled

the minimal effective strain (MES). When load-induced strains exceed the MES, a primary signal is generated within the tissue and perceived by bone cells. In response, bone cells contribute to the generation of a secondary signal that alters bone remodeling and modeling (Figure 2-1). In this case, remodeling would be inhibited while the addition of new bone mass via the modeling process would be increased. Adaptive changes in skeletal mass should then act as a feedback mechanism to alter the MES.

Since both the addition and removal of loads affect bone mass, Frost suggested that the effects of strain on skeletal homeostasis could be viewed via four mechanical usage windows (198). In the adapted window, mechanical strains are within the MES and the activation of remodeling occurs at a normal rate while modeling is essentially turned 'off'. In the disuse window, strains fall below the MES and bone loss ensues. Modeling remains 'off' or is decreased while the activation of remodeling events increases with bone resorption exceeding bone formation. Conversely, in the mild overload window, strains exceed the MES and an increase in modeling leads to new lamellar bone formation and the MES being reset. Finally, in the pathological overload window bone experiences loads sufficient to damage the tissue. The activation of remodeling varies in response to the need to repair microdamage, while modeling increases bone formation, but woven bone is deposited. Table 2-1 lists the differences between these four windows and theoretical strains for each window.

Some authors have suggested that age-related osteoporosis may be related to a failure of bone's adaptive response to mechanical loads (199). Others suggest that since osteoporosis does not refer to a single disease, one mechanism is unlikely (200).

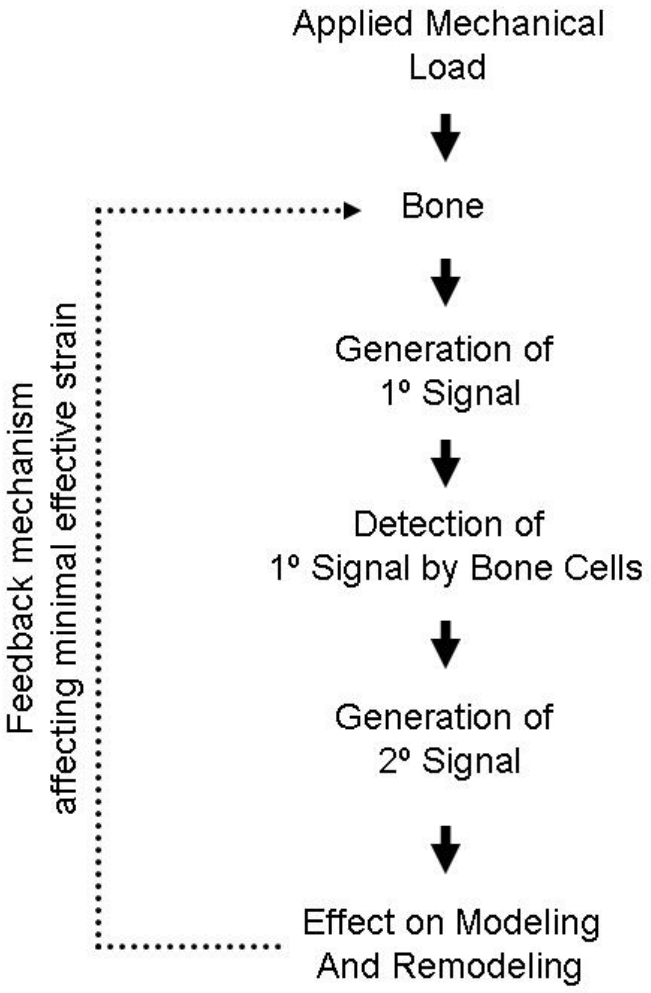

Figure 2-1 Frost's mechanostat theory
After Frost (196,197).

Table 2-1 Mechanical usage windows

	Disuse	Adapted	Mild Overload	Pathological Overload
Remodeling activation	↑ Activation	Normal activation	Normal activation	Variable activation
Remodeling Effect	↑ Resorption ↓ Deposition	Resorption ≈ Depostion	Resorption ≈ Depostion	Resorption ≈ Depostion
Modeling	↓ Modeling	No Modeling	↑ Modeling Lamellar bone deposited	↑ Modeling Woven bone deposited
Strain Range	< 50μ*E*	50-1500μ*E*	1500-3000μ*E*	> 3000μ*E*

After Frost (198)

2.2 Potential Load-induced Signals Detected by Bone Cells

Many investigators and a number of studies have attempted to identify the primary signal that mechanical loads induce within skeletal tissue, and is perceived by bone cells. For the purposes of this thesis, the focus will be on the two most extensively studied stimuli: substrate strain and interstitial fluid flow.

2.2.1 Substrate strain

Substrate strain, resulting from the deformation of skeletal tissue, is a candidate for the load-induced signal perceived by bone cells. Osteocytes, encapsulated within the bone matrix, or osteoblasts, lining the bone surfaces, may detect strains in the bone matrix via direct cellular deformation. Indeed, *in vitro* studies suggest that substrate strain increases osteoblast proliferation and expression of bone matrix proteins (201,202). One problem with these studies is that the levels of substrate strain necessary to generate a response are supra-physiologic. While vigorous physical activity induces strains of 500-2500μ*E in vivo* (203,204), studies suggest that strain magnitudes 5- to 10-fold higher are necessary to alter gene expression (201,202). For example, exposing MC3T3-E1 osteoblasts to substrate strains less than 5000μ*E* failed to enhance the expression of osteopontin mRNA or induce the mobilization of intracellular calcium (205,206). Strains of this magnitude are associated with tissue damage and fracture.

2.2.2 Interstitial fluid flow

Emerging data suggest that bone cells may instead act as fluid sensors and respond to mechanical loading through the detection of interstitial fluid flow (207). The deformation of skeletal tissue in response to applied loads induces the pressurization of interstitial fluid and stimulates its movement along pressure gradients, from the matrix

into haversian systems (4). Due to the cyclical nature of physiological loading, interstitial fluid flow is likely to be oscillatory in nature. In a normal gait cycle, loading, which drives fluid out of the matrix, is followed by a period of relaxation, thus reversing the pressure gradient and allowing interstitial fluid to flow back into the matrix (Figure 2-2).

Two proposed mechanisms by which bone cells may perceive interstitial fluid flow are via fluid shear stress or chemotransport effects. Within the confined geometry of bones lacuanar-canilicular and haversian systems, it is predicted that fluid flow imparts shear stresses upon the cell membrane estimated to be between 8 and 30dynes/cm^2 (5,208,209). *In vitro* studies indicate that the predicted levels of shear stress are potent regulators of bone cell behavior, as fluid flow enhances the expression of several phenotypic markers of osteoblastic cells, including osteopontin (206,210) and type I collagen (211), and enhances cellular proliferation (212,213). Alternatively, interstitial fluid flow may mediate bone's adaptive response to mechanical loads by enhancing chemotransport mechanisms between the blood supply and bone tissue (4). *In vitro*, two studies suggest that chemotransport effects contribute to bone cell mechanotransduction. Haut Donahue *et al*. (6) found that a decrease in the flow rate at a fixed fluid shear stress decreased the percentage of cells responding to fluid flow with an increase in intracellular calcium concentration. Additionally, Allen *et al*. (214) found that serum was necessary for cells to respond to fluid flow. The relative contribution of chemotransport and fluid shear stress remains controversial and may depend upon the fluid flow regime utilized (170,215).

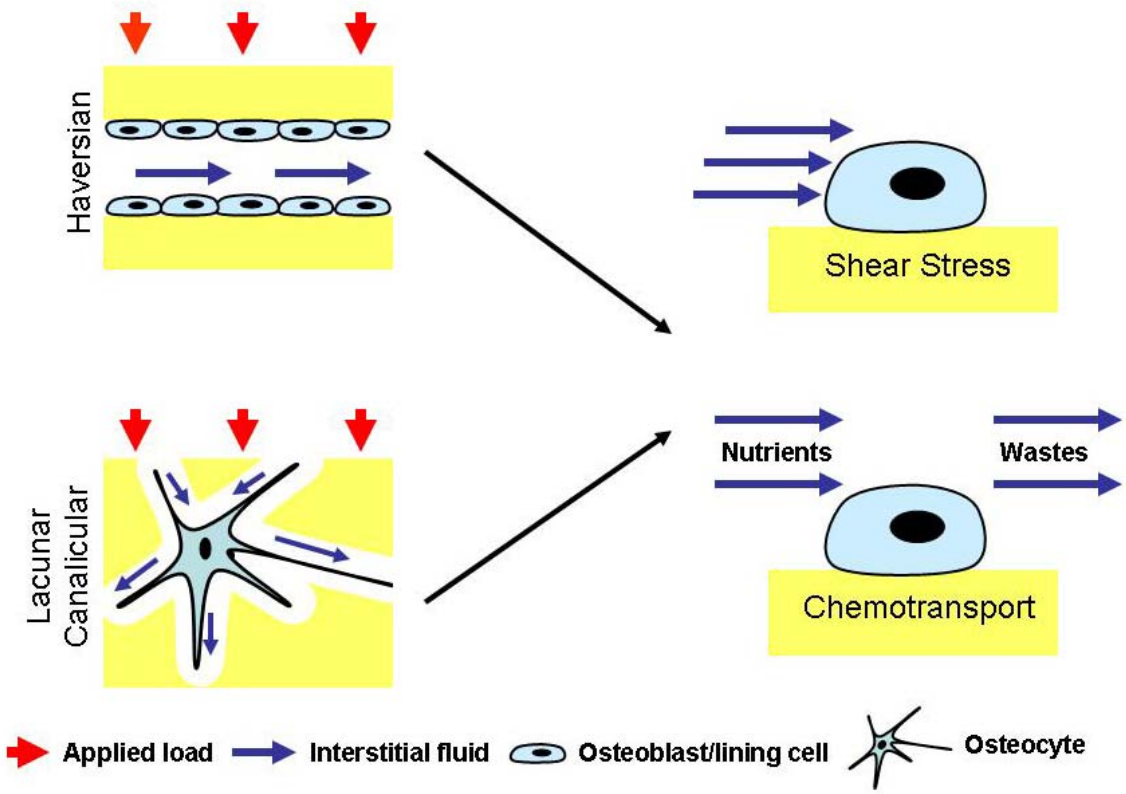

Figure 2-2 Interstitial fluid flow and detection by bone cells. The application of exogenous loads leads to the pressurization of interstitial fluid and its movement along pressure gradients in bone spaces like haversian systems and the lacunar-canalicular system. Interstitial fluid flow may be detected by bone cells in the form of fluid shear stress or chemotransport effects.

2.3 Candidate Mechanosensors

The exact mechanism by which cells convert mechanical stimuli into a biochemical response remains elusive, but several potential mechanosensors have been postulated. These include the cell membrane, integrin/cytoskeletal interactions, ion channels, and connexin hemichannels.

2.3.1 Cell membrane

Direct pertubations of the cell membrane may represent one mechanism by which cells perceive changes in their mechanical environment. In endothelial cells, fluid flow exposure induces an increase in membrane fluidity that may lead to the activation of intracellular signaling events (216,217). For instance, increasing membrane fluidity can contribute to the activation of ion channels (218,219). Additionally, exposing phospholipid vesicles containing G proteins to fluid flow leads to GTP hydrolysis, a measure of G protein activity, and this response can be modulated by altering membrane fluidity with cholesterol or benzyl alcohol (220). Mechanistically, it is believed that the increase in membrane fluidity allows for the dissociation of the α-subunit from βγ and subsequent hydrolysis of GTP independent of a G protein coupled receptor. In osteoblasts, membrane fluidity also contributes to the ability of cells to sense fluid flow. However, this is thought to be mediated by alterations in lipid raft and caveolae formation (221).

The cell membrane might also act as a mechanosensor via the glycocalyx. Many cell types, including bone cells, are covered by a layer of hyaluronic acid and other proteoglycans (222,223). Fluid flow might produce drag forces on the glycocalyx that bone cells perceive (5). Consistent with this hypothesis, Reilly *et al* (224) reported that

enzymatically degrading the glycocalyx abrogated the release of PGE_2 after fluid flow stimulation.

2.3.2 Integrins and the cytoskeleton

The activation of integrin signaling and transmission of biophysical signals to the cytoskeleton represents another possible mechanism by which cells perceive changes in their mechanical environment. Similar to the glycocalyx, You *et al* (225) postulated that drag forces on the pericellular matrix in response to fluid flow could be coupled to and amplified by the actin cytoskeleton. Consistent with this hypothesis, the pericellular space surrounding osteocytes within the lacunar-canalicular is filled by matrix. Tethering elements, presumably composed of integrins, connect the canalicular wall and pericellular matrix to the cytoskeleton (226). A few *in vitro* studies also implicate integrins and the cytoskeleton in the sensitivity of bone cells to fluid flow. Pavalko *et al*. (227) reported that disrupting the actin cytoskeleton with cytochalasin D or inhibiting integrin/actin filament interaction abolished the increase in cyclooxygenase-2 and c-fos in response to fluid flow. Additionally, fluid flow induces the recruitment of the adaptor molecule Shc to $\alpha_v\beta_3$ integrins, suggesting the activation of integrin signaling (228).

2.3.3 Ion channels

Electophysiology studies reveal that bone cells express a number of mechanosensitive ion channels (229) and these channels could represent a mechanism by which bone cells respond to fluid flow. The opening of stretch-activated channels may lead to the direct activation of intracellular signaling components or lead to the depolarization of the cell membrane and subsequent activation of voltage-sensitive channels. Duncan and Hruska (230) reported that mechanical strain stimulates stretch-activated channel activity that

could be inhibited with gadolinium. Later, fluid flow was found to open similar channels as well as L-type calcium channels (231). Importantly, inhibition of L-type channel activity with nifedipine or verapamil or stretch-activated channels with gadolinium suppresses load-induced bone formation (232,233).

2.3.4 Connexin hemichannels

Recent evidence suggests that connexin hemichannels also represent a putative mechanosensor. The opening of these unopposed gap junction channels would allow for the passage of small signaling molecules from the cytosol to the extracellular spaces (234). In astrocytes and glioma cells, mechanical stimulation leads to the release of ATP from connexin hemichannels (235,236). Studies from our laboratory and others suggest that hemichannels are activated by fluid flow in osteocytic cells. Exposing MLO-Y4 cells, a model for osteocytes, to fluid flow increases the recruitment of connexin 43 to the plasma membrane and the opening of hemichannels as assessed by the uptake of fluorescent tracers (237-239). Once activated, hemichannels mediate the release of paracrine factors that activate intracellular signaling pathways.

2.4 Components of Bone's Mechanotransduction Pathways

While the exact mechanosensory mechanism remains unknown, a number of cellular responses to fluid flow have been identified. These include the release of autocrine/paracrine factors, the activation of calcium signaling pathways, and the activation of protein kinase signaling pathways.

2.4.1 Autocrine/paracrine factors

The release of autocrine/paracrine factors is a vital and well established response to fluid flow. These factors can be divided into two groups: those that are released immediately after fluid flow exposure and those that require synthesis.

Recent evidence suggests that the release of ATP and subsequent activation of purinergic receptor is an immediate response to fluid flow. Genetos *et al*. (7) reported that exposing MC3T3-E1 preosteoblasts to fluid flow stimulated a robust increase in ATP release with levels in conditioned media samples peaking within one minute of the initiation of fluid flow. Exposing an osteocytic cell model to fluid flow also induced ATP release (239). Whereas ATP release from osteoblasts occurs via a vesicular mechanism (7,240,241), release from osteocytes occurs via connexin hemichannels (239). The expression of purinergic receptors has been reported for many bone cell types (242) and disrupting their expression affects bone cell mechanotransduction. You *et al*. found that the activation of the $P2Y_2$ receptor induces an increase in intracellular calcium concentration in response to fluid flow, while Li *et al*. (10) reported that the $P2X_7$ receptor partially mediates the anabolic response of bone to mechanical loads.

With longer term exposure to fluid flow, bone cells release two other factors: PGE_2 and nitric oxide (NO) (8,170,243,244). These responses require alterations in gene expression, namely an increase in the expression of cyclooxygenase-2 (227,245) and endothelial nitric oxide synthase (246,247). The specific effects of PGE_2 on bone cell behavior are discussed in Chapters 1 and 3. NO contributes to osteoblast proliferation and differentiation by inducing MAP kinase signaling (213), and may also be anti-

apoptotic (248). Pharmacological inhibition of PGE_2 or NO production *in vivo* inhibits bone formation after the addition of exogenous loads (249-253).

2.4.2 Calcium signaling

An increase in intracellular calcium concentration is one of the earliest responses of bone cells to fluid flow (254) and requires both the entry of extracellular calcium and the release of calcium from intracellular stores (255). As mentioned above, osteoblasts and osteocytes express a number of calcium-conducting channels, including L- and T-type calcium channels and an unidentified mechanosensitive cation channel (230,256,257), that are activated by fluid flow. Exposing cells to fluid flow in calcium-free media or pharmacological inhibition of these channels dampens the elevation of intracellular calcium in response to fluid flow (210,255,258). Calcium signaling is similarly affected when intracellular calcium stores are emptied or phospholipase C activity is inhibited (210,255,258).

The increase in intracellular calcium concentration is reported to contribute to the enhanced expression of osteopontin (210), c-fos, cyclooxygenase-2 (258), and transforming growth factor-β (259), but there is little data on how this change in ion concentration results in altered gene expression. There is evidence that calcineurin (260,261) and calmodulin-dependent kinase (262,263) regulate osteoblast proliferation and differentiation, but a clear link between fluid flow and the activation of these proteins has not been established.

2.4.3 Protein kinase signaling

The activation of a number of protein kinase signaling cascades has been noted in response to fluid flow. Of these, mitogen activated protein kinase signaling has been the

most thoroughly studied. Exposing osteoblastic cells to fluid flow stimulates the phosphorylation and activation of extracellular signal-regulated kinase-1 and -2 (ERK1/2) as well as p38 (210,211,264). The activation of these kinases contributes to a number of endpoints following fluid flow exposure. You *et al*. (210) reported that ERK1/2 and p38 activation mediates the enhanced expression of osteopontin in response to fluid flow, while Wadhwa *et al*. (264) suggest ERK1/2 signaling contributes to the induction of cyclooxygenase-2 expression by fluid flow. Additionally, many studies have found that ERK1/2 activation is necessary for fluid flow to stimulate osteoblastic cell proliferation (212,213,265). Activation of protein kinase C and protein kinase A have also been reported in response to fluid flow (266-268), and may also be involved in the induction of cyclooxygenase-2 expression and PGE_2 release.

CHAPTER 3

MESENCHYMAL STEM CELLS

Stem cells, with the capacity for self-renewal and multi-lineage differentiation, play a vital role in tissue homeostasis and repair. As such, stem cells have been isolated from a number of tissues including skin (269,270), adipose (271,272), and muscle (273,274). The bone marrow contains two populations of stem cells: hematopoietic stem cells that give rise to all blood cell lineages (275,276) and mesenchymal stem cells, the focus of this thesis. Identifying extracellular cues that control the proliferation and lineage determination of these cells could have important implications for the treatment of skeletal disorders as well as tissue engineering practices.

3.1 Discovery and Multi-lineage Potential

Alexander Friedenstein and colleagues are generally credited with the discovery of bone marrow-derived mesenchymal stem cells. Implanting suspensions of bone marrow cells under the renal capsule of mice stimulates the formation of a new bone unit complete with a marrow compartment (277). Whereas the cells composing the bony elements are derived from donor tissue, the hematopoietic cells are derived from the recipient (278). Later, the ability to regenerate a bone organ was attributed to a population of adherent, clonogenic cells labeled colony forming unit-fibroblastic (CFU-F) (279-281).

New evidence suggests that the CFU-F has the potential to support the differentiation of lineages beyond that of the osteoblast. A large number of studies have reported that this cell population supports differentiation along the osteogenic, chondrogenic, adipogenic and myogenic lineages, as well as hematopoietic-supporting stroma (282-286). Figures 3-1 and 3-2 illustrate the osteoblastic and adipocytic differentiation of the cells used in this thesis. Other studies suggest these cells can also differentiate to

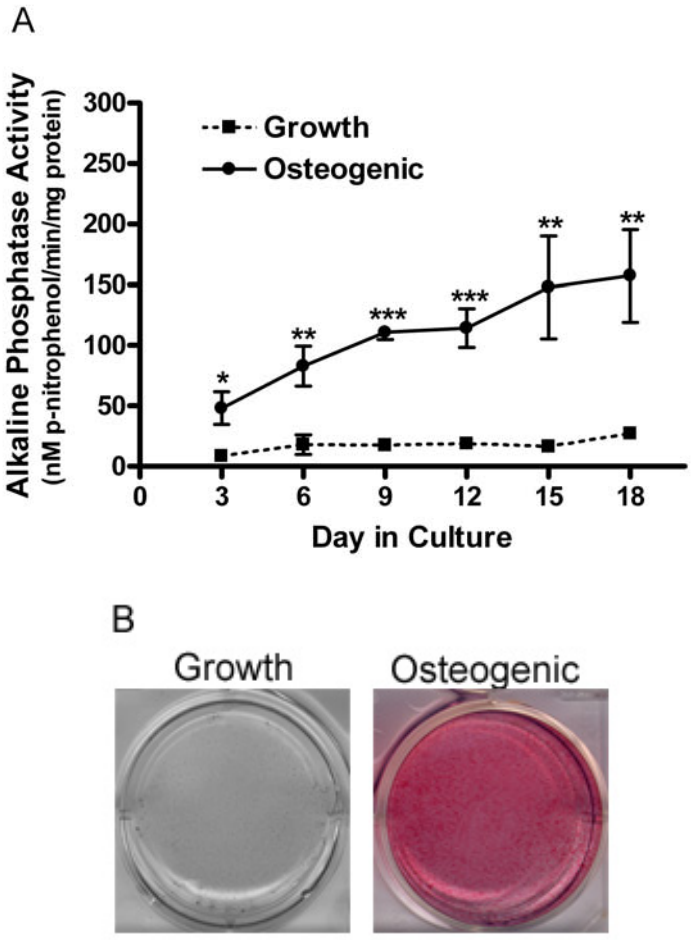

Figure 3-1. Osteoblastic differentiation of hMSCs. Cells were cultured in growth media (DMEM-low glucose containing 10% fetal bovine serum and 2mM L-glutamine) or osteogenic media (growth media supplemented with 10nM dexamethasone, 10mM β-glycerophosphate, and 50μg/ml ascorbic acid phosphate). Levels of alkaline phosphatase activity were assessed on days 3-18 (A). Calcium deposition was assessed on day 18 by staining with alizarin red (B). *$p<0.05$, **$p<0.01$, ***$p<0.001$ to growth media control.

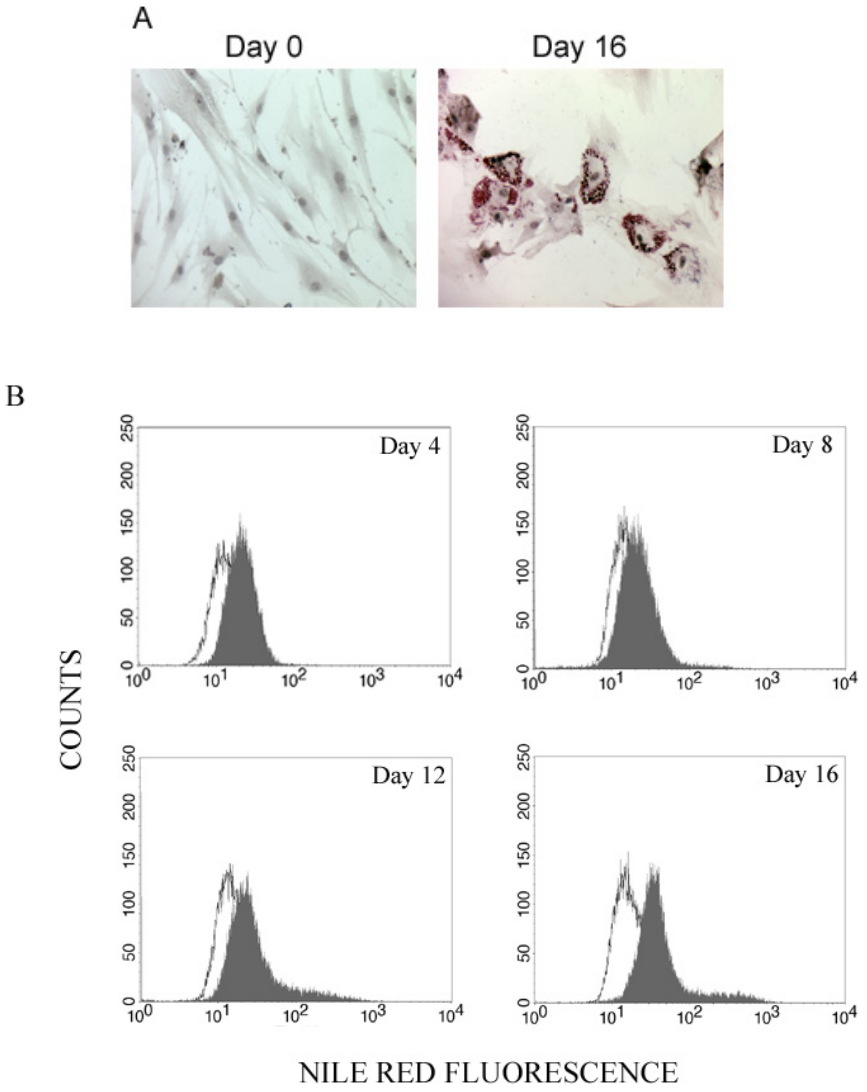

Figure 3-2. Adipogenic differentiation of hMSCs. Cells were sequentially treated with media supplemented with 10µg/ml insulin, 1mM dexamethasone, 100µg/ml IBMX, and 50µg/ml indomethacin for three days followed by 10µg/ml insulin for one day for 16 days. Lipid accumulation was assessed by staining with Oil Red O on day 16 (A). (B) Changes in lipid accumulation through this time course were assessed by fluorescent-activated cell sorting using nile red. Clear populations represent cells on day 0, while the shaded populations represent adipogenic cells on the indicated culture day. Note the appearance of more differentiated cells on days 12 and 16.

lineages as diverse as astrocytes, oligodendrocytes, and neurons (287-289). As a result, the CFU-F is routinely referred to as a bone marrow stromal cell, marrow stromal stem cell, or mesenchymal stem cell (MSC).

Unfortunately the protocols routinely used in the isolation of this cell population result in a great deal of heterogeneity, and some authors refrain from calling the CFU-F a stem cell (290,291). As described by Friedenstein (279-281), most studies isolate MSCs based on their tissue-culture plastic adherence (described in (292)). Using this method, Owen *et al*. (293) reported a wide variability in the differentiation potential of individual colonies, with some lacking osteogenic potential entirely. Similarly, when Kuznetsov *et al*. (294) derived strains of human MSCs (hMSCs) from single cells, only 60% of strains could form bone *in vivo*. Those cells not able to form bone likely represent a more committed progenitor cell. To combat this heterogeneity, many studies have attempted to identify MSC-specific cell surface markers. Gronthos *et al*. (295) used the marker STRO-1 (296) to identify the fraction of human bone marrow with the potential for osteoblastic differentiation. This marker is highly expressed by MSCs and declines as cells acquire alkaline phosphatase expression (297). Other studies have utilized Thy-1 (298), endoglin (299), SSEA-4 (300), and the α_1 integrin subunit (301). To date, an MSC-specific marker has not been identified.

3.2 Pathological Alterations in MSC Proliferation and Differentiation

Accumulating evidence suggests that alterations in MSC plasticity may be related to the development of osteoporosis and other metabolic bone diseases. Many conditions that lead to a loss of bone mass, including ovariectomy (125,302), spinal cord injuries (303), and glucocorticoid treatment (304), are associated with an increase in marrow

adiposity. Similarly, age-related changes in bone mass are accompanied by an increase in the number and size of marrow adipocytes (305,306). Given the osteoblastic and adipogenic differentiation potential of MSCs, a current and well accepted hypothesis suggests an increase in bone marrow adipocytes occurs at the expense of osteoblast differentiation (307).

Both i*n vitro* and *in vivo* data support this hypothesis. Comparing the differentiation potential of MSCs isolated from young (4months) and old (24months) BALB/c mice, Bergman *et al*. (308) observed a 41% decrease in the number of osteogenic colonies formed by cells derived from older mice. Additionally, MSCs isolated from older mice exhibited an attenuated response to a proliferative signal. Rodriguez *et al* (309) observed similar defects in proliferation and differentiation in MSCs isolated from postmenopausal women with osteoporosis compared to normal controls. The SAMP6 mouse is a model of accelerated senescence and is osteopenic (310,311). Compared to "normal" aging controls, MSCs isolated from this model exhibit a three-fold decrease in formation of alkaline phosphatase colonies, while exhibiting a significant increase in the ability to form adipogenic colonies (312). In humans, the proportion of bone marrow cavity occupied by adipocytes is inversely related to the rate of bone formation (313).

McCune-Albright syndrome (MAS) represents another bone disease associated with alterations in MSC behavior (314). MAS or fibrous dysplasia of bone results from an activating mutation in the α subunit of the stimulatory G protein, Gs (315,316), and leads to the replacement of the marrow cavity with a fibrous tissue and the formation of abnormal and mechanically-inferior skeletal structures (317). The observation that bone cells isolated from MAS patients exhibit an increased proliferation rate and pre-

osteoblastic phenotype, led Riminucci *et al* to hypothesize that this disease results from changes in the differentiation and function of MSCs (318,319). Indeed, transplanting mutant MSCs in immuno-compromised mice recapitulated this disease (320).

Taken together these data strongly suggest that identifying factors that regulate the proliferation and osteoblastic differentiation of MSCs, and possibly inhibit adipogenic differentiation (321), will provide a valuable therapeutic target in many bone diseases.

3.3 Therapeutic Potential of MSCs

Due to their multipotent differentiation capacity there has been a great deal of interest in utilizing these cells for gene therapy and tissue engineering (322-324). Many studies have already revealed the capacity of MSCs to migrate to and engraft in multiple organs (287,325,326). The engraftment of MSCs, isolated from healthy donors, in the bone marrow of children with osteogenesis imperfecta stimulates an increase in growth and bone mineral density while decreasing fracture risk (327,328). These data suggest that MSC transplants may be an effective treatment for this condition. MSCs might also be effective in the repair of cardiovascular (329), lung, (330), and spinal cord (331,332) injuries. However, care must be taken in the interpretation of these results to ensure that tissue repair is mediated by MSC differentiation and not cell fusion (333). Additionally, scaffolding materials designed to enhance the osteogenic capacity of MSCs while filling orthopaedic defects and enhancing tissue integration are currently under development (334-337).

One problem associated with the use of this cell type in therapeutic applications is that they represent only a small fraction of the cell present in bone marrow. Pittenger *et al*. (284) estimated that MSCs represent only 0.001 to 0.01% of the cells in marrow

aspirates following density gradient centrifugation. While these cells can be propagated *in vitro*, two significant concerns exist. First, *in vitro* expansion is associated with a decrease in proliferation and differentiation capacity (338,339). Second, the use of fetal bovine serum to expand cell populations *in vitro* may elicit an immune response when cells are transplanted *in vivo* (340,341). Thus, the identification and thorough understanding of the factors which regulate the behavior of these cells will be vital for the successful use of MSCs in therapeutic practices.

A number of the factors that direct MSC proliferation and differentiation are described below.

3.4 Transcriptional Regulators of MSC Differentiation

Figure 3-3 illustrates the major transcription factors regulating MSC differentiation.

3.4.1 Osteoblastic differentiation

A number of transcription factors have been identified that regulate the commitment of multipotent cells to the osteoblast lineage including CBFA1 and Osterix.

Using the mouse osteocalcin promoter, Ducy and Karsenty (342) identified two osteoblast-specific *cis*-acting elements. OSE1 bound a nuclear factor, expressed by osteoblasts prior to mineralization, while OSE2 bound a nuclear factor expressed at all stages of osteoblastic differentiation. Sequence analysis of the OSE2 element revealed homology to the binding site for Cbfa/AML/PEBP2α/Runx2 transcription factors and the factor binding this element was labeled CBFA1/OSF2 (343). Expression of this protein is first detected in the mesenchymal condensations prefiguring the skeleton and is restricted to cells of the osteoblastic lineage (344). The skeletons of mice lacking CBFA1 fail to mineralize owing to a complete lack of mature osteoblasts (345,346),

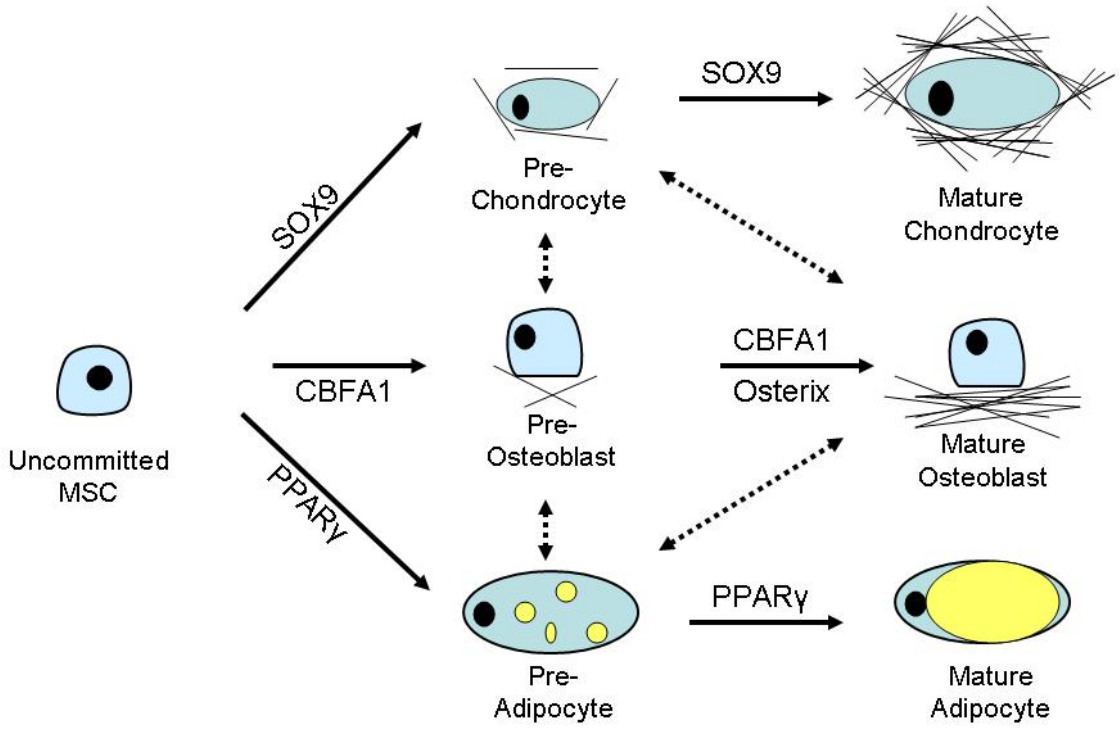

Figure 3-3 Major transcriptional regulators of MSC differentiation.

while the phenotype of heterozygotes is characteristic of Cleidocranial dysplasia (347). Further studies revealed that CBFA1 is also necessary for sustained osteoblastic activity as postnatal expression of a dominant-negative protein results in osteopenia and abolishes the expression of osteocalcin, osteopontin and bone sialoprotein (348). Subsequently, binding sites for CBFA1 were identified in the promoter regions of type I collagen (349), osteoprotegerin (350) and collagenase-3 (351). Since its discovery, numerous interacting proteins (352-358), hormones (359-361), growth factors (362,363), and signaling cascades (364-367) that regulate CBFA1 transcriptional activity have also been identified.

Using a differential hybridization screen, Nakashima *et al*. (368) identified a second osteoblast specific transcription factor, Osterix/sp7. Similar to mice lacking CBFA1, mice lacking Osterix fail to form bone due to an arrest in osteoblast development. Expression levels of type I collagen are dramatically reduced compared to wild-type embryos, while osteopontin, and bone sialoprotein are not expressed. The fact that mesenchymal cells from Osterix-null mice still express CBFA1, suggests that this factor is genetically downstream of CBFA1. Indeed, overexpression studies in murine embryonic fibroblastic cell line C3H10T1/2 as well as chondrocytic ATDC5 cells, suggest that CBFA1 controls the expression of Osterix (369). Treating hMSCs with BMP-2 or IGF-I induces Osterix expression and induces osteoblastic differentiation (370,371).

Numerous other transcription factors with more restricted roles in osteoblast differentiation have also been identified. ATF4, a member of the CREB family of transcription factors, binds to OSE1 and mice lacking this factor exhibit a delay in

ossification and are osteopenic (372,373). Cooperative interaction between ATF4 and CBFA1 controls the expression of osteocalcin (374). Mice lacking the homeobox proteins Msx1 or Msx2 exhibit a delay in cranial bone formation (375) possibly through a decrease in progenitor cell differentiation (376). Twist proteins interact with CBFA1 and inhibit its activity (377). Importantly, in all of these cases, functioning osteoblasts are still present.

3.4.2 Adipogenic differentiation

Using the mouse aP2 promoter, Graves *et al* (378) and Ross *et al* (379) identified a *cis*-acting element which conferred adipocyte-specific gene expression and the factor that binds this element was labeled ARF6 (adipocyte restricted-factor 6) (380). Later, the factor was identified as a heterodimer composed of the retinoid X receptor and peroxisome proliferator-activated receptor γ (PPARγ) (381,382). Binding sites for PPARγ have been found in the regulatory regions of several adipose related genes including lipoprotein lipase (383) and phosphoenolpyruvate carboxykinase (384). Importantly, overexpressing PPARγ in fibroblastic cells induces an adipogenic phenotype (385), while transfecting more determined myoblasts with PPARγ and CCAAT/enhancer-binding protein-α induces the transdifferentiation to adipocytes (386). Numerous natural and synthetic PPARγ ligands which activate its transcriptional activity have been identified (387-390).

The hypothesis that the adipogenic differentiation of MSCs is regulated by PPARγ stems partially from the observation that thiazolidinediones, PPARγ ligands used to increase insulin sensitivity, stimulate fat accumulation in the bone marrow cavity (391,392). Subsequent *in vitro* studies revealed that these agents stimulated the

adipogenic differentiation of MSCs (393) while inhibiting osteoblast differentiation (394,395). Indeed, PPARγ interacts with and inhibits the transcriptional activity of CBFA1 (396,397). *In vivo* studies have produced similar results as administration of rosiglitazone decreases bone mineral density and osteoblast number while increasing the number of adipocytes within the bone marrow (398). Along these same lines, heterozygous PPARγ deficiency leads to an increase in osteoblastogenesis in MSC cultures and a high bone mass phenotype in mice (399). Further, genetic ablation of PPARγ expression in white adipose tissue increases osteoblast formation to the point that hematopoiesis relocalizes to the spleen as in osteopetrosis (400).

3.4.3 Chondrogenic differentiation

The chondrogenic differentiation of multipotent MSCs is regulated by the transcription factor Sox9 (401). Much like the identification of CBFA1 and PPARγ, this transcription factor was identified due to its ability to bind to a *cis*-acting element that conferred cell lineage specific gene expression. Using the first intron of the murine Col2a1 gene, two groups identified a 48 basepair element necessary for cartilage specific gene expression (402-405). Later, the transcription factor binding this element was identified as Sox9 (406-408), a factor strongly expressed in mesenchymal condensations prefiguring the skeleton and during chondrocyte differentiation (409). Similar Sox9 binding sites have also been identified in the promoter regions of the chondrocyte-marker genes aggrecan (410) and Col11a2 (411). Genetic deletion of Sox9 prior to mesenchymal condensation results in a lack of both cartilage and bone, while deletion after this time-point results in chondrodysplasia and a lack of chondrocyte development (412). Conditions that induce the chondrogenic differentiation of MSCs, such as hypoxia (413),

TGF-β or BMP treatment (414,415), and activation of Wnt signaling (416,417), do so by increasing the transcriptional activation of Sox9.

3.5 Paracrine Regulators of MSC Proliferation and Differentiation

3.5.1 Wnt signaling

In recent years, there has been a great deal of interest in the effects of Wnt signaling on skeletal homeostasis as mutations in the Wnt co-receptor LRP5 have been linked with accrual of peak bone mass (418,419). These factors regulate both the proliferation and differentiation of MSCs. Specifically, treating hMSCs with dickkopf-1, a Wnt signaling inhibitor, enhances cellular proliferation (420,421). Wnt3a enhances MSC migration (422) but inhibits the osteoblastic differentiation of MSCs (423), while Wnt10B promotes osteoblastic differentiation at the expense of adipogenic differentiation (424). Additionally, the interaction of Wnt signaling and other growth factors has been shown to both promote and inhibit MSC differentiation (425-427).

3.5.2 Growth factors

A myriad of growth factors that enhance the proliferation and differentiation of MSCs have been identified. IGF-I stimulates the proliferation of MSCs and acts synergistically with TGF-β and BMP-2 to enhance chondrogenesis and osteogenesis, respectively (370,428). FGF-2 inhibits the osteoblastic differentiation of MSCs but can be used to maintain multipotency during *in vitro* expansion (429,430). EGF and PDGF both stimulate MSC migration (431,432). Taken together, these data suggest that growth factors could be used to harness the therapeutic potential of MSCs.

3.5.3 Prostaglandins

As suggested in Chapter 1, the effects of prostaglandins on bone mass may be partially related to the effects of these molecules on MSCs. Following the systemic administration of PGE_2, MSCs isolated from rats exhibit an enhanced capacity for osteoblastic differentiation (433). Cells in these cultures exhibited elevated levels of alkaline phosphatase and formed a greater number of mineralized nodules. Pharmacological evidence suggests that this effect is mediated by the EP_4 receptor (185). These results have important implications for the fracture healing process in which MSCs migrate to sites of skeletal injury (434). Mice lacking COX-2, the rate-limiting enzyme in prostaglandin synthesis, exhibit a delay in fracture healing compared to wild-type controls due to alterations in MSC differentiation (435). Rather than differentiate to osteoblasts at the fracture site, MSCs form a fibrous matrix that results in a non-union. PGE_2 treatment rescues the deficit in osteoblastic differentiation and fracture healing in COX-2-/- mice. Interestingly, systemic administration of PGE_2 may also be able to abrogate the age-related decline in the osteoblastic differentiation of MSCs (436).

3.6 Matrix Regulation of Proliferation and Differentiation

The extracellular matrix and integrin binding may play an important role in regulating MSC behavior. Mauney *et al*. (437-439) have reported that MSCs cultured on denatured collagen during *in vitro* propagation maintain their differentiation potential longer than those cultured on tissue culture plastic. Whereas MSCs cultured on tissue culture plastic gradually lose their potential to express alkaline phosphatase and deposit calcium, cells cultured on denatured collagen retain this capacity with prolonged culture. Additionally, culturing MSCs on matrices composed of fibronectin, vitronectin, collagen type I, or

laminin-5 enhances osteoblastic differentiation compared to standard tissue culture plastic (440-443). In support of these data, mice lacking β_2 integrin are osteopenic and MSCs isolated from these animals exhibit an impaired ability to undergo osteoblastic differentiation and form a mineralized matrix (444).

3.7 Mechanical Signals as Regulators of Proliferation and Differentiation

Emerging evidence suggests that like osteoblasts and osteocytes, MSCs are responsive to mechanical signals and that such signals may contribute to the proliferation and differentiation processes of these cells. Following hindlimb suspension, an accepted model of skeletal unloading, MSCs isolated from rats exhibited a significant decrease in proliferation potential (445) as well as an impaired differentiation capacity (446,447). Similar results have been observed in MSCs isolated from mice (448). Reloading partially rescues deficits in MSCs proliferation and differentiation (449). In humans, skeletal unloading is associated with an increase in bone marrow adipocity which may represent an alteration in MSC differentiation (303). However, these studies do not address the possibility that mechanical signals are communicated to MSCs by more mature bone cell types (49,51).

Recently, the direct effects of biophysical signals on MSC behavior have been examined *in vitro*. Exposing MSCs to cyclic strain during osteogenic differentiation enhances the expression of the osteogenic marker alkaline phosphatase (450) as well as the degree of matrix mineralization (451). Similarly, fluid flow exposure stimulates MSC proliferation (452) and enhances the expression of osteocalcin, osteopontin, and bone sialoprotein (452-454), while the removal of mechanical loads via modeled microgravity impairs the ability of MSCs to express similar markers (455,456). Taken together these

studies suggest that MSCs are individually responsive to biophysical signals. Unfortunately, the cellular mechanisms by which MSCs respond to mechanical stimuli have yet to be defined.

3.8 An Appropriate Biophysical Signal for MSCs

As presented in chapter 2, mechanical loads produce a diverse set of biophysical signals that bone cells may perceive and as such, it is unclear what biophysical signals MSCs are exposed to *in vivo*. We hypothesize that, like osteocytes embedded in the bone matrix, MSCs are exposed to fluid flow in at least three conditions. First, physiological loading is associated with an increase in intramedullary pressure that drives interstitial fluid from the marrow cavity into the bone matrix (457-459). This change in pressure and ensuing fluid flow is associated with bone formation and abolishes bone loss in experimental models of disuse (460,461). In this situation, MSCs localized to confined marrow space could be exposed to significant shear stresses, while others experience significant chemotransport. Second, MSCs with osteogenic potential have been isolated from the circulation (462,463) which would suggest that these cells could localize to haversian systems. Theoretical modeling performed by Swan *et al*. (209) suggests that fluid flow in these vessels would induce fluid shear stresses similar to those experienced by osteocytes within the lacunar-canalicular system (5). Third, Parfitt (89) suggested that the arrival of fibroblastic cells, indicative of MSCs, precedes the appearance of osteoblastic cells in a remodeling osteon. Load-induced fluid flow through a remodeling osteon may regulate the proliferation and subsequent differentiation of MSCs to bone-forming osteoblasts (464,465).

Thus, the purpose of this thesis is to examine the effects of oscillatory fluid flow on hMSC proliferation. The specific aims are to identify the signaling cascades necessary for fluid flow to enhance hMSC proliferation, to examine the signal that initiates the activation of these signaling events, and to identify the biophysical signal by which hMSCs perceive changes in their mechanical environment. These studies may help to explain the changes in MSC proliferation associated with aging and skeletal unloading, and may lead to the development of novel protocols designed to control MSC behavior in tissue-engineering practices.

CHAPTER 4

MAP KINASE AND CALCIUM SIGNALING MEDIATE FLUID FLOW-INDUCED PROLIFERATION

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Mechanical signals are important regulators of skeletal homeostasis and strain-induced oscillatory fluid flow is a potent mechanical stimulus. While the mechanisms by which osteoblasts and osteocytes respond to fluid flow are being elucidated, little is known about the mechanisms by which bone marrow-derived mesenchymal stem cells respond to such stimuli. Here, we show the intracellular signaling cascades activated in human mesenchymal stem cells by fluid flow are similar to those activated in osteoblastic cells. Oscillatory fluid flow inducing shear stresses of 5, 10, and 20dynes/cm^2 triggered rapid, flow rate-dependent increases in intracellular calcium that pharmacological studies suggest are IP_3-mediated. The application of fluid flow also induced the phosphorylation of extracellular-signal regulated kinase-1 and -2 as well as the activation of the calcium-sensitive protein phosphatase calcineurin in mesenchymal stem cells. Activation of these signaling pathways combined to induce a robust increase in cellular proliferation. These data suggest that mechanically-induced fluid flow not only regulates osteoblastic behavior but also regulates that of mesenchymal precursors, implying that the observed osteogenic response to mechanical loading may be mediated by alterations in the cellular behavior of multiple members of the osteoblast lineage, perhaps by a common signaling pathway.

4.1 Introduction

Maintenance of appropriate bone mass requires the coordination of bone resorption by osteoclasts and bone deposition by osteoblasts, and it is well established that mechanical stimuli can regulate the balance between bone formation and resorption. The addition of exogenous mechanical load is believed to stimulate new bone formation through increases in osteoblastic activity and concomitant decreases in osteoclastic

activity (3). Conversely, removal of mechanical load, as is the case during space flight and disuse, leads to decreased osteoblastic activity and loss of bone mass (192,466). Accumulating evidence suggests that individual bone cells, including osteocytes and osteoblasts, are responsible for perceiving and responding to mechanical signals. Such signals, which may include streaming potentials, mechanical strain, and fluid shear stress, elicit a host of biochemical responses including mobilization of second messengers such as calcium (210,254), nitric oxide (9,244), prostaglandins and inositol triphosphate (467); activation of kinase cascades including the mitogen-activated protein kinase and protein kinase C pathways (267,468); and modulation of gene expression (205,227,258). Strain-induced oscillatory fluid flow has been shown by our laboratory and others to be a potent biophysical stimulus (205,206). In MC3T3-E1 preosteoblasts, fluid flow induces the mobilization of intracellular calcium (Ca^{2+}_i), activates extracellular-signal regulated kinase-1 and -2 (ERK1/2) and increases osteopontin mRNA levels (6,205,210). Recent evidence has shown that exposing the osteocytic cell line MLO-Y4 to physiological levels of fluid flow induces similar responses (224,469,470).

While the mechanisms by which osteoblasts and osteocytes respond to mechanical stimuli are being elucidated, little is known about how bone marrow-derived mesenchymal stem cells (MSCs) respond to mechanical signals. Capable of regenerating bone (277,471), these cells also maintain the capacity to differentiate to multiple mesenchymal cell types including chondrocytes and adipocytes (284,472). Emerging studies suggest that, like osteoblasts and osteocytes, MSCs are mechanoresponsive. MSCs isolated from rats following hindlimb unloading exhibit both a decreased proliferative potential and a reduced osteogenic capacity when compared to loaded

controls (445,446). Specifically, cells from these animals formed fewer mineralized and alkaline phosphatase-positive colonies *in vitro*, two commonly used markers of osteoblastic potential. Similarly reduced osteogenic capacity was observed in MSCs isolated from mice subjected to periods of tail-suspension (448). More recently, Simmons *et al.* (451) showed that exposing human mesenchymal stem cells (hMSCs) to a cyclic strain during osteoblastic differentiation enhances the degree of matrix mineralization, and Li *et al*. (452) showed oscillatory fluid flow stimulates hMSC proliferation *in vitro*.

Despite these observations, the cellular mechanisms by which MSCs respond to mechanical stimuli have yet to be defined. In these studies, we sought to investigate whether human marrow-derived progenitors respond to oscillatory fluid flow in a manner similar to that observed in more mature cells of the osteoblastic lineage. Specifically, we hypothesized that fluid flow would result in an increase in intracellular calcium concentration and the subsequent activation of downstream signaling proteins, such as ERK1/2 and calcineurin, which would in turn induce hMSCs proliferation. $Ca^{2+}{}_i$ is a vital and ubiquitous mediator in the processes by which extracellular signals are conveyed to the cell's interior and translated into a cellular response. Oscillations in $[Ca^{2+}]_i$ regulate gene expression via numerous signaling cascades (473,474) and have been shown to provide specificity among transcriptional activators (475,476). The serine/threonine protein phosphatase calcineurin, for instance, responds to increases in $[Ca^{2+}]_i$ and calmodulin binding by dephosphorylating and activating NFAT transcription factors (477,478). Likewise ERK1/2, members of the mitogen-activated protein kinase

family, have been shown to be key regulators in the proliferation and differentiation of numerous cell types including hMSCs (479) and osteoblasts (265,480,481).

Here, we show that exposing hMSCs to oscillatory fluid flow induces a flow-rate dependent increase in $[Ca^{2+}]_i$ and that the release of calcium from IP_3-sensitive stores contributes to this increase. Additionally, fluid flow stimulates a robust and time-dependent increase in ERK1/2 phosphorylation that is independent of the release of $Ca^{2+}{}_i$, but dependent upon the activity of phospholipase C (PLC) and protein kinase C (PKC). Finally, we show that oscillatory fluid flow induces a proliferative response in hMSCs that is dependent upon the activation of ERK1/2 and the activation of calcineurin.

4.2 Materials and Methods

4.2.1 Cell culture

Human mesenchymal stem cells from an 18 year old, male donor (Cambrex Biosciences) were cultured in Dulbecco's modified Eagles medium-low glucose (DMEM-LG; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 2mM L-Glutamine, and 1% penicillin and streptomycin and maintained in a humidified incubator at 37°C with 5% CO_2. Media was replaced every 3-4 days. Supplements to induce the differentiation of hMSCs were not added to the culture medium at anytime in these studies. At 80% confluence, cells were subcultured and seeded on quartz slides for $[Ca^{2+}]_i$ experiments or glass slides for ERK1/2 phosphorylation, calcineurin activity, and proliferation studies. 80,000cells were seeded on quartz slides (76 x 26 x 1.6mm), while 130,000cells were seeded on glass slides (75 x 38 x 1.0mm) to attain similar levels of confluence on the day of experiments. 48hours after seeding, culture media was replaced with flow media (DMEM-LG containing 0.5% FBS) and cells were cultured for an additional 24hours before exposure to fluid flow.

4.2.2 Application of fluid flow

To expose cells to fluid flow slides were positioned in parallel plate flow chambers (482) and connected to a servopneumatic materials testing device (EnduraTec) via glass Hamilton syringes and rigid wall tubing. For calcium imaging experiments a chamber with a fluid volume of 34 x 10 x 0.28mm was employed, while a larger chamber with a fluid volume of 75 x 34 x 0.28mm was used for long-term experiments. The relationship between flow rate, which was monitored in real-time with an ultrasonic flowmeter (Transonic Systems), and shear stress was calculated using the equation

$$\tau=6Q\mu/bh^2 \quad \text{(Equation 1)}$$

where τ is the wall shear stress (dyne/cm^2); Q is the flow rate (cm^3/s); μ is the viscosity of the flow media (0.01dynes·sec/cm^2); and b and h are the width and height of the channel (482). We utilized a flow regime that facilitates the oscillatory movement of a defined volume of fluid across the cell monolayer. Oscillatory fluid flow mimics the shear stresses associated with the loading and unloading of long bones during normal gait, and as such was implemented at a physiological frequency of 1Hz (i.e. 1 step/sec)(5). Flow rates of 4.5, 9, and 18ml/min were used to apply physiologically relevant shear stresses of 5, 10, and 20dynes/cm^2 for calcium imaging experiments, while flow rates of 14, 28, and 56ml/min were used for long-term flow experiments.

4.2.3 Calcium imaging

Calcium imaging experiments were performed as described previously (11,210). Briefly, 80% confluent cells were washed with flow media and incubated at 37°C for 30min in a 10μM fura-2-acetoxymethyl ester (Molecular Probes) solution. Cells were then rinsed with flow media, mounted on a parallel plate flow chamber and placed on an

inverted fluorescent microscope (Nikon). Fura-2 is a Ca^{2+}-responsive fluorescent dye that exhibits a shift in absorption when bound to Ca^{2+}. The emission intensity following ultraviolet illumination increases with $[Ca^{2+}]_i$ at 340nm and decreases with $[Ca^{2+}]_i$ at 380nm. Consecutive images at 340nm and 380nm were recorded once every 2s and analyzed using Metafluor image analysis software (Universal Imaging, West Chester, PA). Absolute $[Ca^{2+}]_i$ is obtained by comparison to a calibration curve provided by the manufacturer (Molecular Probes). Basal $[Ca^{2+}]_i$ was sampled for 3min to serve as a static control and was followed by 3min of oscillatory fluid flow.

4.2.4 Western immunoblotting

To examine the effect of fluid flow on ERK1/2 phosphorylation, hMSCs were exposed to oscillatory fluid flow with a shear stress of 20dynes/cm^2 for 1 to 120min, or 5 and 10dynes/cm^2 for 15min or used as time-matched static controls. Immediately after exposure to flow, cells were washed in ice-cold phosphate buffered saline and total cellular protein isolated in 0.1% Triton X-100, 10mM Tris pH 8, 1mM EDTA, 0.2mM Na_3VO_4, supplemented with a protease inhibitor cocktail (Calbiochem). 25μg of protein were resolved by SDS-PAGE (Gradipore) and transferred to PVDF membranes (BioRad). Increases in ERK1/2 activation in response to fluid flow were assessed by immunoblotting for the phosphorylated forms of ERK1/2 with a specific antibody (Cell Signaling Technology). Immunoreactive bands were visualized by enhanced chemiluminescent detection (Amerisham Biosciences). Blots were subsequently stripped in 0.1M Glycine pH 2 and reprobed for total ERK1/2 (Santa Cruz Biotechnology). Densitometric analysis was carried out using Quality One image analysis software (Bio-rad).

4.2.5 Calcineurin activity assays

The effect of fluid flow on cellular calcineurin activity levels was assessed using a commercially available, colorimetric assay (Calbiochem) according to the manufacturer's instructions. Briefly, cells were exposed to oscillatory fluid flow as described for ERK1/2 experiments and immediately washed in Tris-buffered saline to avoid phosphate contamination. Cell lysates were collected in 50mM Tris, 1mM DTT, 100μM EDTA, 100μM EGTA, and 0.2% NP-40, and then incubated with the RII phosphopeptide (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu) in the presence or absence of 10mM EGTA to inhibit calcineurin activity. Calcineurin activity was calculated by subtracting phosphatase activity in the presence of EGTA from total phosphatase activity. Results are normalized to total protein concentration (BCA, Pierce) and presented as pmol phosphate liberated/mg protein in 30min.

4.2.6 Cellular proliferation assays

Oscillatory fluid flow-induced hMSC proliferation was assessed using a FITC BrdU Flow kit (BD Pharmigen) according to the manufacturer's instructions. Briefly, cells were exposed to oscillatory fluid flow with shear stresses of 5, 10, or 20dynes/cm^2 for 1h or used as static controls. Cells were post-incubated for 20 hours in fresh flow media at 37°C with 5% CO_2. Proliferating cells were then labeled for 60min with 10μM 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue incorporated into the genomic DNA of proliferating cells. Cells were subsequently washed with PBS, fixed, and the percentages of proliferating cells quantified by fluorescence-activated cell sorting (BD, FACScans) using a FITC-conjugated antibody specific for BrdU.

4.2.7 Pharmacological inhibitors

To ascertain the source of fluid flow-induced $[Ca^{2+}]_i$ increases and to elucidate the signaling cascades responsible for flow-induced proliferation, a series of pharmacological inhibitors of Ca^{2+} and MAP kinase signaling were employed. Thapsigargin (1μM), verapamil (5μM), and gadolinium chloride (10μM, all from Sigma), were used to inhibit Ca^{2+} release, L-type voltage-sensitive calcium channel activity, and mechanosensitive cation channel activity, respectively (230,483-485). Ryanodine (1μM, Calbiochem) was used to activate and hold open ryanodine-sensitive channels on intracellular calcium stores (486). U73122 (10μM, Calbiochem) was used to antagonize the activity of PLC and thus the production of IP_3 and DAG, while U73343, a weak PLC antagonist was used as a negative control (258,487). GF109203X (1μM, Biomol) and calphostin C (1μM, Calbiochem) were used to antagonize PKC activity (488,489). U0126 (10μM, Cell Signalling Technologies) and cyclosporin A (5μg/ml, Calbiochem) were used to inhibit ERK1/2 activation and calcineurin activity and to examine their roles in fluid flow-induced proliferation (451,490,491). Cells were pretreated with inhibitors or vehicle controls 30min prior to and for the duration of fluid flow. For calcium imaging experiments, inhibitors were added after cells were loaded with Fura-2. All pharmacological inhibitors were dissolved in DMSO, with the exception of verapamil and gadolinium chloride. Verapamil was dissolved in ethanol, while gadolinium chloride was dissolved directly in the flow media. Solvent concentrations in the flow media were maintained at 0.1% (v/v).

4.2.8 Statistical analysis

All experiments were replicated on at least 3 separate days. Increases in $[Ca^{2+}]_i$ during the static period or in response to fluid flow were determined by calculating the average $[Ca^{2+}]_i$ and the standard deviation for individual cells before exposure to flow. A calcium concentration greater than the average $[Ca^{2+}]_i$ plus 4 standard deviations in response to flow or during the static period was defined as a response. No statistical difference was observed between vehicle controls for calcium experiments (one-way analysis of variance), so all controls were combined. Due to variation between proliferation experiments, data were analyzed and expressed as the percentage of static control. All data are expressed as mean ± SEM. One-way analysis of variance and Tukey's multiple comparisons tests were utilized to compare groups. A student's t-test was used to compare calcineurin activity at each time-point examined (Prism, GraphPad Software Inc.). $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Oscillatory fluid flow induces $[Ca^{2+}]_i$ increases

Exposing hMSCs to oscillatory fluid flow resulted in a rapid, yet transient increase in $[Ca^{2+}]_i$ that was flow rate-dependent. Calcium traces from a typical experiment in which hMSCs were exposed to a shear stress of 20dynes/cm^2 are shown in Figure 4-1A. During the static period, 1.4±0.6% of cells exhibited a spontaneous increase in $[Ca^{2+}]_i$ with an amplitude of 68.9±9.1nM (Figure 4-1B, C). A significantly higher percentage of cells exhibited increases in $[Ca^{2+}]_i$ when hMSCs were exposed to oscillatory fluid flow inducing shear stresses of 5, 10, and 20dynes/cm^2 at 1Hz (56±2.4%, 87±7.9%, and 89±10.4%, respectively). Interestingly, the amplitude of the observed $[Ca^{2+}]_i$ increase in response to 5 and 10 dynes/cm^2 (60.0±10.2nM and 96.8±6.3nM) was not statistically

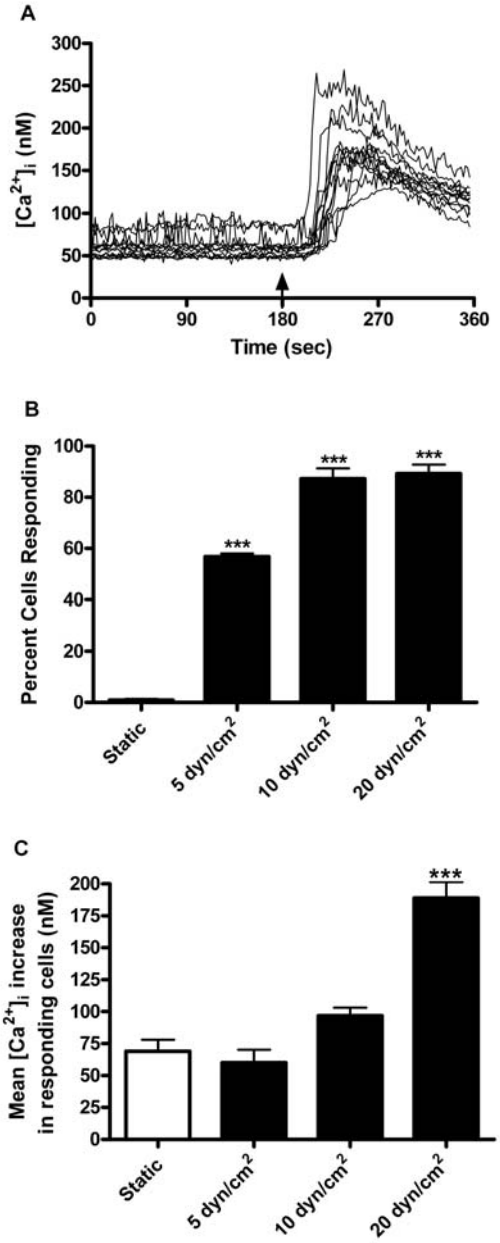

Figure 4-1 Oscillatory fluid flow induces $[Ca^{2+}]_i$ increases in hMSCs. (A) calcium traces from a typical experiment in which hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 at 1Hz. Each line represents the $[Ca^{2+}]_i$ in a single cell, while the arrow represents the onset of flow. (B) percentage of hMSCs responding to oscillatory fluid flow inducing shear stresses of 5, 10, and 20dynes/cm^2. (C) mean increase in $[Ca^{2+}]_i$ in hMSCs responding to fluid flow. *** $p<0.001$ compared to static control.

significant from spontaneous increases observed in static controls. In contrast, a significantly greater increase in $[Ca^{2+}]_i$ was observed when cells were exposed to a shear stress of 20dynes/cm^2 (189.0±12.4nM). Unless otherwise stated, subsequent flow experiments were performed with a shear stress of 20dynes/cm^2 as it produced the most robust increase in $[Ca^{2+}]_i$.

4.3.2 Source of $[Ca^{2+}]_i$ increase

Pharmacological inhibitors were employed to elucidate the source of fluid flow-induced $[Ca^{2+}]_i$ increases. Treatment with $GdCl_3$ or verapamil, which antagonizes the activity of the mechanosensitive cation channel and the L-type calcium channel, respectively, did not have a significant effect on either the percentage of cells responding to fluid flow with an increase in $[Ca^{2+}]_i$ (Figure 2A) or flow-induced $[Ca^{2+}]_i$ increases (Figure 2B) when compared to untreated, flow controls. Thapsigargin (1μM), which empties intracellular calcium stores by inhibiting the ATP-dependent Ca^{2+} pump, significantly decreased both the percentage of responding cells (15.5±6.4%) and flow-induced increases (54.6±8.6nM) when compared to flow controls. Flow-induced increases were similar to those observed in static controls (73.8±9.9nM). Thus, we decided to further examine the mechanisms by which fluid flow induces the release of calcium from intracellular stores.

Ryanodine (1μM), which activates and holds open ryanodine-sensitive channels present on intracellular calcium stores, did not have a significant effect on either the percentage of responding cells (88.2±4.4%) or increases in $[Ca^{2+}]_i$ (127.4±9.2nM). However, inhibiting the production of IP_3 by antagonizing the activity of PLC with

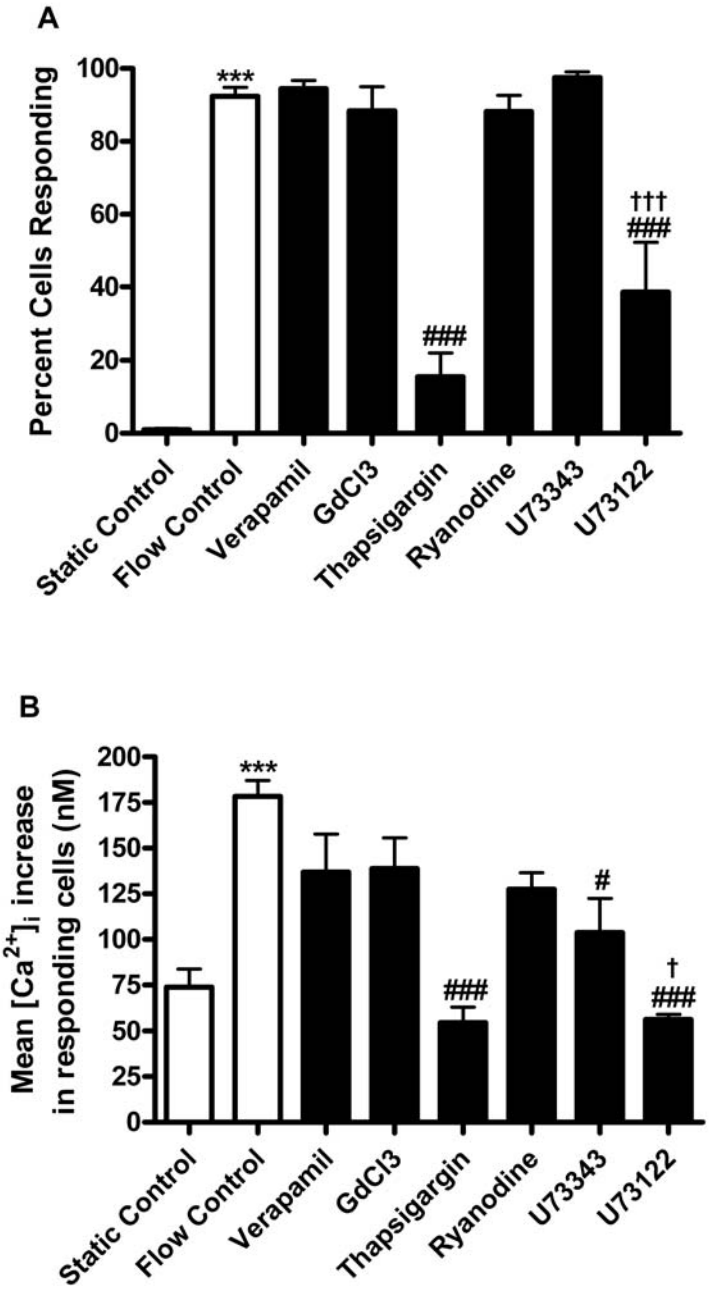

Figure 4-2. Calcium-release contributes to flow-induced transients. (A) percentage of cells exhibiting an increase $[Ca^{2+}]_i$ and (B) mean increase in $[Ca^{2+}]_i$ in response to oscillatory fluid flow (20 dynes/cm^2, 1Hz) in flow controls or in the presence of 5μM verapamil, and 10μM $GdCl_3$, 1μM thapsigargin, 1μM ryanodine, 10μM U73343 or 10μM U73122. *** $p<0.001$ compared to static control. # $p<0.05$, ### $p<0.001$ compared to flow control. † $p<0.05$, ††† $p<0.001$ compared to U73343.

U73122 significantly decreased both the percentage of cells responding to fluid flow (38.6±13.6%) and flow-induced increases in $[Ca^{2+}]_i$ (56.3±2.8nM). U73343 slightly attenuated the increase in $[Ca^{2+}]_i$ in response to fluid flow (103.8 ± 18.7nM), probably as a result of its weak ability to inhibit PLC. However, U73343 treatment did not affect the percentage of cells responding (97.5±1.5%).

4.3.3 Fluid flow induces ERK1/2 phosphorylation

Antibodies specific for ERK1/2 phosphorylated at Thr202 and Tyr204 were used to examine increases in ERK1/2 activation in response to fluid flow. Basal levels of ERK1/2 phosphorylation remained constant in static controls (Figure 4-3A). In response to the onset of oscillatory fluid flow inducing a shear stress of 20dynes/cm^2, we observed a time-dependent increase in ERK1/2 phosphorylation. Following 15, 30, and 60min of fluid flow, phosphorylation was markedly increased compared to time-matched static controls. However, after 120min of fluid flow, phosphorylation levels were comparable to those of unflowed cells. To assess how quickly ERK1/2 phosphorylation levels increased, we repeated these experiments at earlier timepoints. Phosphorylation levels were increased after just one minute of fluid flow, but the highest levels were achieved after 5 and 15 minutes (Figure 4-3B).

As several studies have noted the importance of $Ca^{2+}{}_i$ signaling in the activation of ERK1/2 in osteoblastic cells (492,493), we investigated the effect of fluid flow-induced $[Ca^{2+}]_i$ increases on ERK1/2 activation. Cells were treated with a series of pharmacological inhibitors 30min prior to and during a 15min exposure to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2. Interestingly, thapsigargin (1μM), which blocked flow-induced $[Ca^{2+}]_i$ increases (Figure 4-2), did not significantly affect

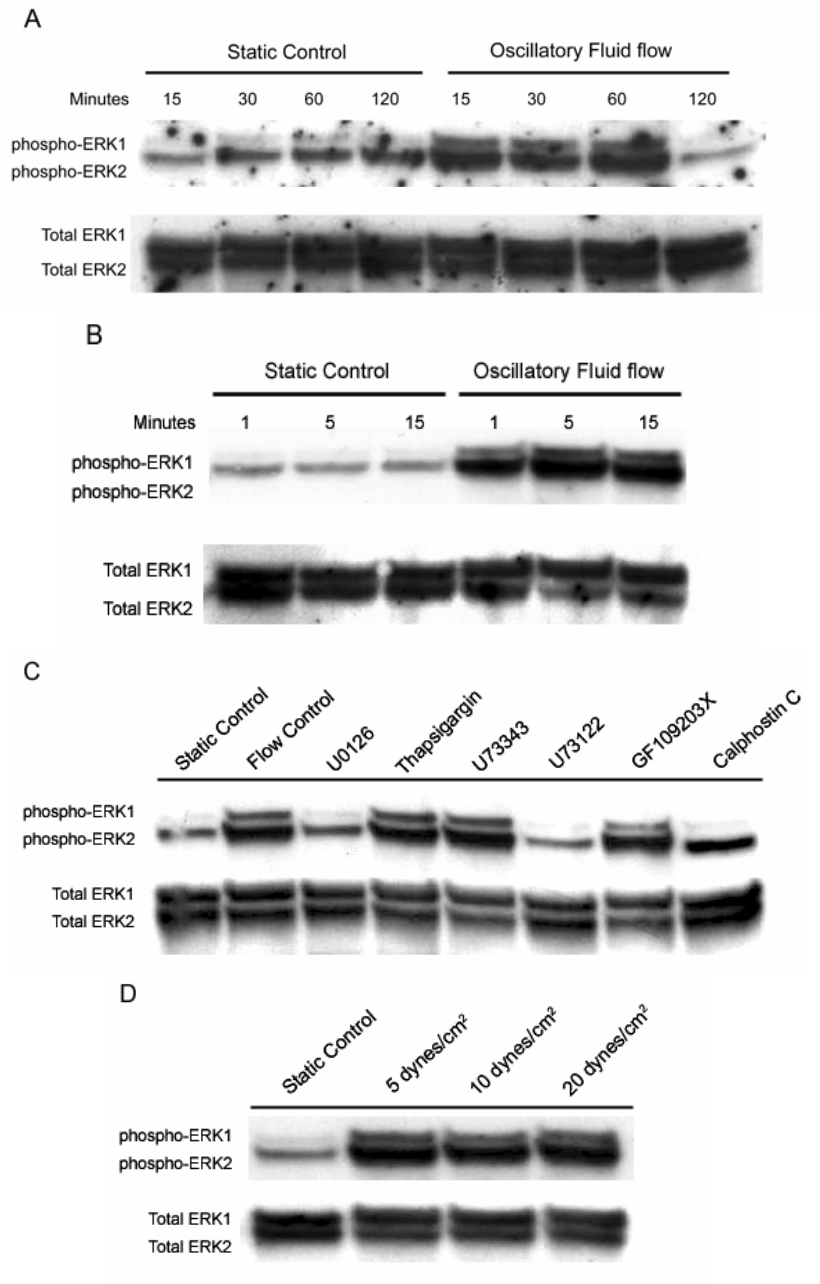

Figure 4-3. Oscillatory fluid flow triggers ERK1/2 phosphorylation. hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 at 1Hz for 15 to 120min (A) and from 1 to 15min (B) or left unflowed as time-matched static controls. ERK1/2 phosphorylation levels were examined by immunoblotting. (C) hMSCs were left unflowed or exposed to oscillatory fluid flow for 15min in the presence of vehicle control, 10μM U0126, 1μM thapsigargin, 10μM U73343, 10μM U73122, 1μM GF109203X or 1μM Calphostin C. Densitometric analysis of these data revealed a 66.3%, 81.8% and 71.6% decrease in the ratio of phospho-ERK1/2 to total ERK1/2 with U0126, U73122, and Calphostin C treatment, respectively. (D) hMSCs were exposed to oscillatory fluid flow inducing shear stresses ranging from 5 to 20dynes/cm^2 for 15min.

ERK1/2 phosphorylation in response to flow (Figure 3C). Flow-induced ERK1/2 phosphorylation was blocked by treatment with U73122 (10μM), which also inhibited $[Ca^{2+}]_i$ (Figure 4-2), but was unaffected by its non-inhibitory analog, U73343. Since these data suggest a role for PKC in the flow-induced phosphorylation of ERK1/2, we treated hMSCs with 1μM GF109203X or 1μM Calphostin C. GF109203X antagonizes most isoforms of PKC, but in these studies it did not affect flow-induced ERK1/2 phosphorylation. In contrast, preventing the binding of DAG to PKC by treatment with Calphostin C attenuated the flow-induced phosphorylation of ERK1/2. The discrepancy between these results may be related to the isoenzyme specificity of PKC inhibitors (488,494). Treatment with the MEK inhibitor U0126 (10μM) also abolished flow-induced increases in ERK1/2 phosphorylation and was used as a positive control. Neither $GdCl_3$ nor verapamil treatment affected the levels of flow-induced ERK1/2 phosphorylation, and we did not observe an effect of GF109203X, Calphostin C, or U0126 treatment on flow induced $[Ca^{2+}]_i$ increases.

Since we observed a flow rate-dependent increase in $[Ca^{2+}]_i$, we examined the possibility that ERK1/2 phosphorylation might also be flow rate-dependent. Our studies showed that ERK1/2 were equally phosphorylated in response to fluid flow with shear stresses of 5, 10, or 20dynes/cm^2 (Figure 4-3D). These data may be a result of signal amplification within the MAP kinase signal cascade or the inherent sensitivity of the pathway to extracellular signals such that all three flow rates induce the same phophorylation response.

4.3.4 Calcineurin activity is increased by fluid flow

Since blocking fluid flow-induced increases in $[Ca^{2+}]_i$ did not prevent ERK1/2 phosphorylation, we examined other possible downstream targets of this response, such as activation of the protein phosphatase calcineurin. Control cells consistently exhibited low levels of calcineurin activity, ranging from 310.6±117.1pmol phosphate liberated/mg protein after 1min to 377.9±76.2pmol phosphate liberated/mg protein after 15min (Figure 4-4). In response to fluid flow, calcineurin activity levels increased significantly to 901.5±101.8pmol phosphate liberated/mg protein after 1min of flow. Activity levels remained significantly elevated through 5 and 15 minutes of fluid flow (788.4±139.8 and 766.4±125.2pmol phosphate/mg protein, respectively).

4.3.5 Fluid flow induces proliferation via ERK1/2 and calcineurin activation

As calcium and MAP kinase signaling have both been implicated in cellular proliferation, we examined the effect of oscillatory fluid flow on hMSC proliferation. hMSCs were exposed to oscillatory fluid flow for 1h and then incubated for 20h before labeling with 10μM BrdU to allow cells the time to synthesize the factors necessary for cell cycle progression. Exposing hMSCs to fluid flow with shear stresses of 5 or 10dynes/cm^2 failed to induce a significant increase in cellular proliferation (Figure 4-5A). However, the highest flow rate examined (20dynes/cm^2) induced a 126.1±16.1% increase in proliferation over static controls.

We next examined the effect of fluid flow-induced ERK1/2 and calcineurin activity on flow-induced proliferation by pretreating cells with 10μM U0126 or 5μg/ml cyclosporin A. Inhibition of ERK1/2 activation did not have a significant effect on basal proliferation levels, but completely abolished flow-induced increases in hMSC

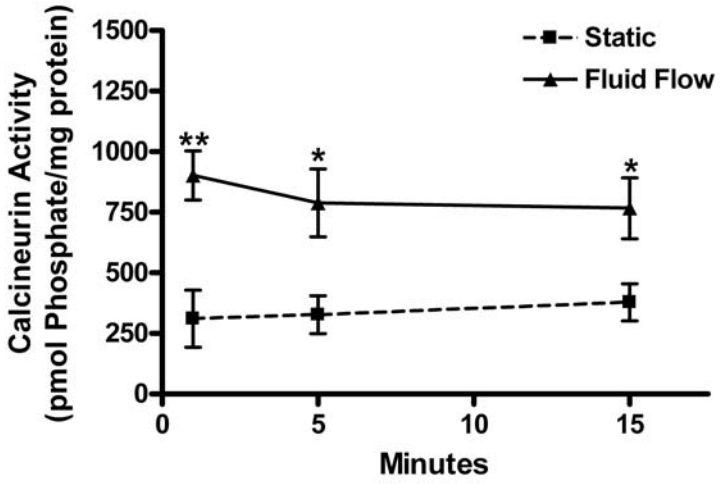

Figure 4-4. Fluid flow increases calcineurin activity levels. hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 at 1Hz for 1 to 15 minutes or used as time-matched static controls. Cellular lysates were incubated with a specific phospho-peptide and liberated phosphate was quantified. * $p<0.05$ compared to static control ** $p<0.01$ compared to static control.

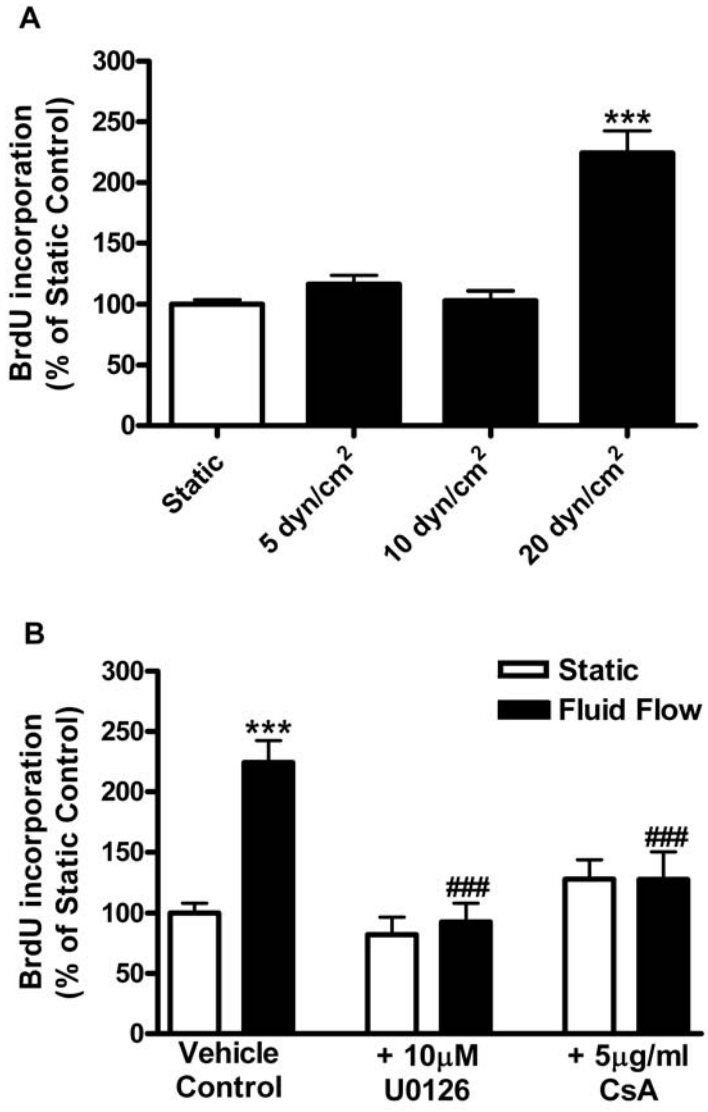

Figure 4-5. Fluid flow induces hMSC proliferation. (A) hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 5, 10, or 20dynes/cm^2 for 1 hour or left unflowed, as static controls, and then returned to culture for 20hours in flow medium and proliferating cells were quantified by FACS analysis. (B) hMSCs were left unflowed or exposed to oscillatory fluid flow (20dynes/cm^2) for 1hour in the presence of vehicle control, 10μM U0126, or 5μg/ml cyclosporin A (CsA). Due to variation between experiments, results are represented as a percentage of the static control. *** $p<0.001$ compared to static control. ### $p<0.001$ compared to flow control.

proliferation (92.9±15.3%, Figure 4-5B). Treatment with cyclosporin A had a similar effect (127.8±22.6%) suggesting that the activation of both of these signaling molecules is necessary for the proliferative response of hMSCs to fluid flow.

4.4 Discussion

Maintenance of skeletal homeostasis is clearly regulated by biophysical signals. Understanding the mechanisms by which individual bone cells perceive and respond to these signals may serve as a starting point for the treatment of conditions in which bone cell behavior is altered. While the mechanisms by which osteoblasts and osteocytes perceive and respond to these signals are under scrutiny, little is known about the mechanism by which MSCs respond to biophysical signals. Accumulating evidence suggests that marrow-derived osteoprogenitors are also mechanoresponsive (445,446,448,450-452) and, as such, understanding the mechanism by which MSCs respond to biophysical signals is as important as understanding that of osteoblasts or osteocytes. MSCs represent a pool of osteoprogenitors and better insight into the factors that regulate their proliferation and differentiation may provide new therapeutic opportunities to ameliorate bone loss.

Several studies have suggested that increased bone formation in response to mechanical loading is mediated by the response of bone cells to strain-induced fluid flow (207,208). The deformation of bone tissue during physiological levels of mechanical load (such as ambulatory motion) induces cyclical movement of interstitial fluid flow, with predicted fluid shear rates calculated to be between 8 and 30dynes/cm^2 (5). Exposing osteoblasts to these shear rates *in vitro* induces the expression of the genes associated with bone formation (210,495) and has been shown to be a more potent

stimulus than directly applying physiologic levels of mechanical strain (205,206). In the present study, we sought to examine the effect of fluid flow on hMSC behavior. To date, all estimates of physiologic levels of load-induced fluid flow in bone have been calculated with reference to osteocytic cells (5) and, because of structural differences between the cellular environment of marrow and bone, these values may not be directly applicable to MSCs. However, as cells residing in the marrow are intimately associated with bone surfaces, especially in trabecular bone, we hypothesize that MSCs will also be exposed to oscillatory fluid flow. To address these issues, we selected three flow rates that encompass the predicted physiologic levels of flow that MSCs may experience.

Like osteoblasts and osteocytes, hMSCs responded to fluid flow with a rapid, flow rate-dependent increase in $[Ca^{2+}]_i$. Absolute increases in $[Ca^{2+}]_i$ in response to fluid flow with shear rates of 5 and 10 dynes/cm^2 were not different from spontaneous oscillations observed in hMSCs during the static period. Intriguingly, hMSCs responded to a shear stress of 20dynes/cm^2 with a significantly more robust $[Ca^{2+}]_i$ increase, on the order of 200nM. While the magnitude of this observed $[Ca^{2+}]_i$ increase is in accordance with the recent study by Li *et al*. (452), it is noteworthy that in previous studies conducted with osteoblastic cell models no difference has been observed between the amplitude of flow-induced increases in $[Ca^{2+}]_i$ and spontaneous oscillations observed in unflowed cells (11,205,210). The activation of PLC contributed to flow-induced calcium responses, as treatment with pharmacologic agents that either emptied intracellular calcium stores or blocked IP_3 production attenuated flow-induced calcium increases. These data reflect previous studies that examined the mechanism by which fluid flow induces $[Ca^{2+}]_i$ increases in osteoblasts (210,258). However, while several studies have

implicated the L-type calcium channel and mechanosensitive cation channel in bone cell mechanotransduction (210,230,232), treating hMSCs with pharmacological antagonists directed against these channels had no significant effect on either fluid flow-induced Ca^{2+}_i oscillations or ERK1/2 phosphorylation. These data are supported by recent electrophysiologic studies suggesting that only a small fraction of hMSCs cells express functional L-type channels (496) and may mark a major difference in the mechanotransduction pathways of the osteoblast and the osteoprogenitor.

Several studies utilizing osteoblastic cells have noted the importance of $[Ca^{2+}]_i$ increases in the activation of the mitogen-activated protein kinases ERK1/2 (492,493). These kinases are vital regulators of osteoblastic proliferation and differentiation and have been shown to mediate flow-induced effects on proliferation (212) and gene expression (210,264). Indeed, a recent study by Kapur *et al.* (265) reported the requirement of both ERK-1 and -2 in the proliferative effect of fluid flow. Oscillatory fluid flow also induced the phosphorylation of ERK1/2 in hMSCs. However, flow-induced ERK1/2 phosphorylation in hMSCs was not dependent upon calcium release as ERK1/2 phosphorylation was only blocked by PKC and PLC antagonists. Rather, our data imply that flow-induced calcium responses are likely to be responsible for the activation of calcineurin. Calcineurin activity levels were dramatically increased after 1min of oscillatory fluid flow exposure and remained elevated through 15min.

While these data suggest that in hMSCs, the calcium and MAP kinase responses are initially independent of one another, their actions appear to converge and induce a proliferative effect in response to oscillatory fluid flow. Fluid flow inducing a shear stress of 20dynes/cm^2 triggered a 126% increase in hMSC proliferation, a response that is

attenuated by inhibiting both the phosphorylation of ERK1/2 and activation of calcineurin with U0126 and cyclosporin, respectively. Previous studies utilizing non-osteoblastic cell models, provide support for this signaling mechanism. MAP kinase and calcineurin signaling have both been implicated in the expression, accumulation, and activity of cyclin D, a protein necessary for the G_1 to S transition in the cell cycle (497-499). Further, both signaling molecules have been implicated in cardiac hypertrophy by co-dependently coordinating cardiac cell growth (500). Our data suggest that ERK1/2 may have a secondary role, relative to calcium signaling, in the mechanism by which oscillatory fluid flow induces proliferation. The rationale for this hypothesis is that ERK1/2 was equally phosphorylated at all flow rates, but proliferation was only evident at a flow rate that caused the most robust increase in $[Ca^{2+}]_i$.

In contrast to the aforementioned study by Li *et al.* (27), which demostrated a 57% increase in hMSC proliferation in response to fluid flow inducing a shear stress of 10dynes/cm^2, we only observed a statistically significant increase in cellular proliferation when cells were exposed to a higher flow rate that induced a shear stress of 20dynes/cm^2. This discrepancy is likely to be due to differences in experimental procedure (ie. serum concentrations) and/or assay sensitivity (FACS vs histological quantification). Since we only observed an increase in proliferation at the flow rate that induced the greatest increase in $[Ca^{2+}]_i$, we hypothesize that a robust calcium response and the activation of downstream signaling molecules, in the presence of activated MAP kinases, may act as the molecular switch to induce hMSC proliferation in response to fluid flow.

Due to the oscillatory nature of the flow regime utilized in these studies, there exists the potential for media moving across the hMSC monolayer to become enriched in

secreted factors and metabolites. There are conflicting reports as to whether such chemotransport may affect bone cell behavior. We previously demonstrated that chemotransport contributes to the responsiveness of osteoblastic cell models to oscillatory fluid flow (6). However, reports utilizing primary bone cells suggest that shear rate plays a more important role in mechanotranduction than chemotransport (170,268). Clearly, further study is necessary to clarify the contribution of both chemotransport and fluid shear stresses in the physiological response of bone to mechanical loading.

While these studies highlight that subtle differences exist between the signaling cascades activated in response to fluid flow in more mature cells of the osteoblastic lineage and hMSCs, when taken together these data suggest that a common mechanotransduction pathway exists by which cells of the osteoblastic lineage respond to oscillatory fluid flow. Calcium transients were blocked by inhibiting calcium release from intracellular stores in hMSCs, as is the case in osteoblastic cell lines, and flow-induced proliferation is dependent upon the activation of ERK1/2 (212,265). An intriguing possibility is that the observed effect of fluid flow on hMSCs is mediated by the release of ATP as has been proposed in osteoblasts (7,241,501). The expression of purinergic receptors by hMSCs (Chapter 5) supports this hypothesis and such receptors have been shown to mediate the response of osteoblastic cells to oscillatory fluid flow (11). Indeed, exogenous ATP has been shown to induce a mitogenic response in MC3T3-E1 preosteoblasts (502). Investigation of this possibility may provide further evidence for a common mechanotransduction pathway and a broader understanding of the mechanism by which fluid flow regulates hMSC activity.

In summary, this study outlines for the first time the intracellular mechanism by which oscillatory fluid flow affects human mesenchymal stem cells. We demonstrated that fluid flow induces an increase in hMSC proliferation via a mechanism that causes an increase in intracellular calcium concentration and activates ERK1/2, presumably through the action of PLC and PKC. These data suggest that fluid flow not only regulates osteoblastic behavior but also that of hMSCs and imply that a common pathway exists by which mechanical signals are translated to cellular responses. Further clarification of the intricacies of this pathway may lead to the development of novel therapeutic techniques designed to enhance the recruitment of osteoprogenitors and ameliorate bone loss.

CHAPTER 5

ATP RELEASE MEDIATES FLUID FLOW-INDUCED PROLIFERATION

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Regulation of bone cell activity by autocrine/paracrine factors is a well established mechanism by which skeletal homeostasis is regulated by mechanical signals. The release of extracellular nucleotides in particular has been shown to induce many of the responses thought to be necessary for load-induced bone formation. In these studies, we examined the effect of oscillatory fluid flow on the release of ATP from hMSCs and the effect of ATP release on hMSC proliferation and intracellular calcium signaling pathways. We found that hMSCs express a number of purinergic receptors necessary to respond to extracellular nucleotides and that fluid flow exposure induced the flow-rate dependent release of ATP. Treating hMSCs with monensin and *N*-ethylmaleimide attenuated ATP release, suggesting a vesicular mechanism. Additionally, we found that exogenous ATP, but not other nucleotides, increased cellular proliferation. Moreover, extracellular ATP was a prerequisite for fluid flow-induced increases in intracellular calcium concentration, activation of calcineurin, the nuclear translocation of NFATc1 and proliferation. These data indicate that ATP regulates not only osteoblastic and osteocytic cell behavior but also that of mesenchymal precursors and support our hypothesis that similar mechanotransduction mechanisms are activated by fluid flow in these cell types.

5.1 Introduction

Optimal skeletal architecture is regulated by mechanical stimulation such that alterations in mechanical load are followed by modification of bone mass and structure (3,193,503). Current hypotheses suggest that the application of mechanical loads result in the deformation of skeletal tissue, generating substrate strains that drive the oscillatory movement of interstitial fluid. Osteocytic and osteoblastic cells are believed to respond to this interstitial fluid flow and act to increase osteoblastic activity and enhance bone

formation (5,208,504). Indeed, *in vitro* studies have shown fluid flow to induce osteoblastic responses thought to be necessary for the anabolic response of bone to mechanical loads (210,495). While the signaling pathways that allow bone cells to perceive and respond to these changes in their extracellular environment are under investigation, they are not yet completely understood.

It is clear that the anabolic response of bone to mechanical load depends upon the release of paracrine/autocrine factors that alter bone cell activity. *In vitro*, fluid flow enhances the production and release of prostaglandin E_2 (PGE_2) from both osteoblasts and osteocytes (243,245,467), whereas the inhibition of PGE_2 production *in vivo* prevents mechanically-induced bone formation (250,251). Accumulating evidence suggests that the release of extracellular nucleotides, such as ATP, may also be important paracrine factors in bone cell mechanotransduction. Mechanical stimulation in the forms of hypotonic swelling and fluid flow, have been shown to induce ATP release from osteoblastic cell models (7,241). Moreover, extracellular ATP mobilizes intracellular calcium (11,505); alters transcription factor activity and gene expression (506,507); and increases the proliferation rate of osteoblastic cells (502,508) through the activation of purinergic (P2) receptors.

To date, several P2 receptor subtypes have been cloned and are divided into two classes based upon their mechanism of action. P2X receptors act as ligand-gated ion channels, while P2Y receptors are metabotropic G-protein coupled receptors (509,510). The expression of P2 receptors by osteoblastic cells has been reported (242,511), and $P2X_7$ knockout mice display distinct skeletal phenotypes, including decreased periosteal bone formation rate and increased trabecular bone resorption, resulting from decreased

sensitivity to mechanical signals (10,512). However, the expression of purinergic receptors by or the effect of extracellular nucleotides on the behavior of human mesenchymal stem cells (hMSCs) has not been thoroughly examined.

hMSCs have the potential to differentiate to multiple cell types including osteoblasts, chondrocytes and adipocytes (284,472), and understanding the factors that regulate the proliferation and differentiation processes of these cells may have important implications for the treatment and prevention of bone loss. A number of studies suggest that hMSC proliferation and differentiation is regulated by changes in their mechanical environment. hMSCs isolated from experimental models of disuse form smaller and fewer osteogenic colonies, indicating a deficit in proliferation potential and osteogenic capacity (445,446). Recent *in vitro* studies have suggested that, like osteoblasts and osteocytes, hMSCs are themselves responsive to mechanical stimulation (450-452,513), but the biophysical signal these cells perceive and respond to is unclear. It is possible that, like osteocytes embedded within the bone matrix, hMSCs are exposed to interstitial fluid flow. Increased intramedullary pressure in response to load drives interstitial fluid from marrow spaces into the bone matrix and is associated with bone formation (457-459). Due to the heterogeneity of trabecular structures, some hMSC localized to confined marrow space could be exposed to significant shear stresses, whereas others may experience significant chemotransport. Alternatively, progenitor cells migrating to sites of bone formation could be localized to Haversian systems and theoretical modeling predicts that flow in this area would induce shear stresses similar to that experienced by osteocytes (5,209).

Given their potential use in tissue engineering (322,323), understanding the effect of biophysical signals on hMSC proliferation and differentiation is critical and providing the correct mechanical environment *in vitro* could lead to the optimization of these protocols and greater success *in vivo*. Indeed, exposing hMSC-containing scaffolds to fluid flow enhances the expression of phenotypic markers of osteoblastic cells as well as mineralized matrix deposition when compared to static cultures (514-516). A thorough understanding of the intracellular pathways activated by fluid flow may further contribute to the optimization of these protocols.

In our previous studies, we have shown oscillatory fluid flow to enhance hMSC proliferation via a mechanism that involves the activation of the extracellular signal-regulated kinase-1 and -2 and the mobilization of intracellular calcium (Chapter 4 (513)). However, the upstream signal(s) that triggers the activation of these signaling cascades remains unknown. Here, we hypothesized that oscillatory fluid flow enhances hMSC proliferation through the release of ATP and activation of purinergic receptors. We show that hMSCs release ATP in response to oscillatory fluid flow via a vesicular mechanism, and that they express a number of purinergic receptors. We also provide evidence that ATP is unique, among nucleotides, in its ablility to induce hMSC proliferation. Moreover, we show that ATP is necessary for fluid flow-induced proliferation, acting to trigger the mobilization of intracellular calcium, activate calcineurin and stimulate the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT).

5.2 Materials and Methods

5.2.1 Cell culture

Human mesenchymal stem cells from an 18year old, male donor (Cambrex Biosciences) were cultured according to standard techniques in Dulbecco's Modified Eagles medium-low glucose (DMEM-LG; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 2 mM L-Glutamine, and 1% penicillin and streptomycin and maintained in a humidified incubator at 37°C with 5% CO_2. Media was replaced every 3-4days and cells were used from passage 3 to 8. At 80% confluence, cells were subcultured and 130,000cells were seeded on glass slides (75 x 38 x 1.0mm) or 80,000cells were seeded on quartz slides (76 x 26 x 1.6mm) for calcium imaging experiments. 48hours after seeding, culture media was replaced with flow media (DMEM-LG containing 0.5% FBS) and cells cultured for an additional 24hours before exposure to fluid flow. Cells were exposed to oscillatory fluid flow in flow media.

5.2.2 Application of fluid flow

hMSCs were exposed to fluid flow oscillating at 1Hz in parallel plate flow chambers (482,517) connected to a servopneumatic materials testing device (EnduraTec) via glass Hamilton syringes and rigid wall tubing. Two types of flow chambers with different fluid volumes were utilized in these experiments. For ATP release, proliferation, calcineurin, and NFAT localization experiments a chamber with a fluid volume of 75 x 34 x 0.28mm was used. For calcium imaging and dye uptake experiments, a smaller chamber with a fluid volume of 34 x 10 x 0.28mm was utilized. The relationship between flow rate and shear stress was calculated with the equation

$$\tau=6Q\mu/bh^2 \quad \text{(Equation 1)}$$

where τ is the wall shear stress (dyn/cm^2); Q is the flow rate (cm^3/s) monitored in real time with an ultrasonic flowmeter (Transonic Systems); μ is the viscosity of the flow medium (0.01dyn·s·cm^{-2}); and, b and h are the width and height of the channel (cm), respectively (482). Flow rates of 14, 28, and 56ml/min were used to induce shear stresses of 5, 10, and 20dynes/cm^2 in experiments utilizing the larger flow chamber, while a flow rate of 18 ml/min was used to induce a shear stress of 20dynes/cm^2 in the smaller flow chamber.

5.2.3 Western immunoblotting

The expression of purinergic receptors by hMSCs was examined by immunoblot. hMSCs grown on glass slides as described for fluid flow experiments were washed in PBS and total cellular protein isolated in 0.1% Triton X-100, 10mM Tris, pH 8, 1mM EDTA, supplemented with a protease inhibitor cocktail (Calbiochem). 25µg of protein were resolved by SDS-PAGE (Gradipore) and transferred to polyvinylidene difluoride membranes (Bio-Rad). Antibodies specific for purinergic receptors and appropriate peptide controls were purchased from Alamone Labs. Immunoreactive bands were visualized via enhanced chemiluminescence detection (Amersham Biosciences).

ERK1/2 phosphorylation levels were examined utilizing an antibody specific for phosphorylation at Thr202 and Tyr204 (Cell Signaling) and standardized to total ERK1/2 levels (Santa Cruz Biotechnologies). hMSCs were exposed to fluid flow for 15min or used as time-matched static controls, washed in ice-cold phosphate buffered saline, and then cell lysates were collected in 1% Triton X-100, 10mM Tris, pH 8, 1mM EDTA, 0.2mM Na_3VO_4, before being resolved by SDS-PAGE.

5.2.4 Luciferin:luciferase determination of ATP concentration

Following fluid flow exposure, conditioned media samples were collected and immediately stored at -80°C until the time of analysis. ATP concentrations in conditioned media samples were determined using a commercially available ATP bioluminescence determination kit (Roche). ATP in 50µl of each conditioned media sample was used by luciferase, as a cofactor to convert D-luciferin, in 50µl of a luciferin-luciferase assay buffer into oxyluciferin and light. The luminescence from each reaction, as measured by a Monolight 3010 luminometer (BD Pharmigen), was then compared to a standard curve created by serially diluting an ATP standard. Duplicate measurements were taken from each conditioned media sample. Control experiments were performed with each pharmacological inhibitor to ensure their presence had no detrimental effect on the reaction. Results were normalized to cellular protein concentration using the BCA method (Pierce).

5.2.5 Lactate dehydrogenase measurements

The presence of lactate dehydrogenase (LDH), a cytosolic enzyme released from damaged cells, was measured in conditioned media samples to assess plasma membrane integrity. LDH levels were quantified using a Cytotox96 Nonradioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. In the presence of LDH, lactate is oxidized to pyruvate, producing NADH that in the presence of diaphorase is used as a cofactor in the conversion of iodonitrotetrazolium into a red formazan product. Absorbances at 490nm, which are proportional to the amount of LDH present in conditioned media samples, were compared between static and fluid flow samples. Cell

lysates collected in 0.1% Triton X-100, 10mM Tris pH 8, 1mM EDTA were used as a positive control for LDH activity.

5.2.6 Cellular proliferation assays

hMSC proliferation was assessed using an FITC BrdU Flow kit (BD Pharmigen). Cells were exposed to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 for 1h or treated with the indicated nucleotide in static experiments, and then post-incubated for 20hours at 37°C with 5% CO_2. Proliferating cells were labeled for 60min with 10μM 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue incorporated into the genomic DNA of proliferating cells. Cells were subsequently washed with PBS, fixed, and the percentages of proliferating cells quantified by fluorescence-activated cell sorting (BD, FACScans) using a FITC-conjugated antibody specific for BrdU.

5.2.7 Assessment of gap junctional communication

A double labeling technique utilizing two cell populations was used to assess gap junctional intracellular communication (50,518). Briefly, hMSCs were seeded on 25mm glass coverslips in the bottom of 6well plates at a density of 5000cells/cm^2 and grown for 48hours before serum starving in flow media for 24hours as for fluid flow experiments. On the day of experimentation a second, donor population of hMSCs was loaded with the cytosolic dye calcein-AM (Molecular Probes) and the lypophilic, membrane dye 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) in a solution composed of 2ml HBSS, 2% BSA, 7μl DiI, 20μl calcein-AM, and 20μl pluronic acid (Molecular Probes) and incubated at 37°C for 30min. Donor cells were then collected with trypsin and resuspended in flow media. Donor cells (2000/coverslip) were then dropped onto the acceptor cell population and incubated for 75min at 37°C. If

functional gap junctions form between a donor cell and acceptor cells, calcein, due to its small molecular weight, passes from the cytosol of the donor cell to the acceptor cell(s). Donor cells remain distinguishable from acceptor cells by the presence of a DiI-labeled membrane. Following the incubation, non-adherent cells were removed by washing with PBS. Coverslips were then mounted on glass slides, positioned on a Nikon fluorescent microscope (Nikon EFD-3; Optical Apparatus) and cell coupling visualized using fluorescein and rhodamine filters.

5.2.8 Dye uptake assay

Uptake of the fluorescent tracer, Lucifer Yellow (Sigma) via gap junction hemichannels was assessed as described previously (237,238). Briefly, hMSCs were cultured at low density (3000cells/cm^2) overnight in chamber-slides or on glass slides to minimize physical contact. On the day of the experiment, cells were washed in flow media and then incubated for 10min in the presence or absence of 5mM EGTA, or exposed to oscillatory fluid flow, in flow media containing 1mg/ml Lucifer Yellow and 1mg/ml Rhodamine Dextran. Due to its small size (0.547kDa), Lucifer Yellow passes through open gap junctions hemichannels, while the Rhodamine Dextran (10kDa), too large to pass through gap junction hemichannels, was used as a negative control. Cells were subsequently washed in HBSS, fixed for 10min in 4% paraformaldehyde, and dye uptake observed via fluorescent microscopy.

5.2.9 Cellular localization of ATP (quinacrine staining)

The antimalarial drug, quinacrine was used to identify intracellular ATP stores. hMSCs grown on glass slides as for fluid flow experiments were labeled for 30min at 37°C with 25μM quinacrine (Sigma) in flow media. Cells were then washed twice in

HBSS, counterstained with Hoechst 33258 (5μg/ml from 10mg/ml stock in PBS), and examined via fluorescent microscopy as described above.

5.2.10 Agonist and pharmacological inhibitors

A series of nucleotide agonists and pharmacological and enzymatic agents (all from Sigma) were used to examine the effect of extracellular nucleotides on hMSC proliferation and the mechanism of fluid flow-induced ATP release. Nucleotides, including ATP, CTP, GTP, TTP, UTP, AMP, ADP, adenosine, and apyrase were dissolved directly in flow media. Monensin (100μM), a sodium ionophore, was used to inhibit the formation of vesicles from the Golgi apparatus (519,520), and dissolved in methanol. *N*-ethylmaleimide (100μM) interferes with NEM-sensitive fusion proteins and prevents the fusion of vesicles with the plasma membrane (521) and was dissolved in methanol. 18-α-glycyrrhetinic acid (AGA, 20μM), dissolved in DMSO, was used to inhibit gap junction intracellular communication and hemichannel activity (522). Cells were pretreated with pharmacological and enzymatic agents 30min prior to fluid flow exposure and remained in the flow media for the length of the experiment. Solvent concentrations in the flow medium were maintained at 0.1% (vol/vol).

5.2.11 Calcium imaging

Calcium imaging experiments were performed as described previously (11,210). hMSCs grown on quartz slides were washed with flow media and incubated at 37°C for 30min in a 10μM solution of the Ca^{2+}-responsive indicator fura-2-acetoxymethyl ester (Molecular Probes). Cells were then rinsed with flow media, mounted on a parallel plate flow chamber and placed on an inverted fluorescent microscope (Nikon). Fura-2 exhibits a shift in absorption when bound to Ca^{2+}, such that the emission intensity following

ultraviolet illumination increases with $[Ca^{2+}]_i$ at 340nm and decreases with $[Ca^{2+}]_i$ at 380nm. Consecutive images at 340nm and 380nm were recorded once every 2s and analyzed using Metafluor image analysis software (Universal Imaging). Absolute $[Ca^{2+}]_i$ was obtained by comparison to a calibration curve provided by the manufacturer (Molecular Probes). Basal $[Ca^{2+}]_i$ were sampled for 3min to serve as a static control and was followed by 3min of oscillatory fluid flow.

To examine the effects of extracellular nucleotides on intracellular calcium levels, hMSCs were seeded to 35mm dishes containing glass coverslips. Cells were loaded with Fura-2 as above and basal $[Ca^{2+}]_i$ sampled for 60sec. Cells were then treated with the indicated nucleotide (25μM) or vehicle control and $[Ca^{2+}]_i$ levels sampled for an additional 60sec.

5.2.12 Calcineurin activity assays

The effect of extracellular nucleotides and fluid flow-induced ATP release on cellular calcineurin activity levels were assessed using a commercially available, colorimetric assay (Calbiochem) according to the manufacturer's instructions. Cells were treated with 0-100μM or exposed to oscillatory fluid flow in the presence or absence of 10U/ml apyrase for 5min and immediately washed in Tris-buffered saline. Cell lysates were collected in 50mM Tris, 0.5mM DTT, 50μM EDTA, 50μM EGTA, and 0.1% NP-40, and then incubated with the RII phosphopeptide (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu) in the presence or absence of 10mM EGTA, to inhibit calcineurin activity. Calcineurin activity was calculated by subtracting phosphatase activity in the presence of EGTA from total phosphatase activity. Results

are normalized to total protein concentration (BCA, Pierce) and presented as pmol phosphate liberated/mg protein over 30min.

5.2.13 Immunofluorescence

hMSCs were exposed to fluid flow or treated with ATP for 30min at 37°C, washed with PBS, and fixed for 5min in ice-cold acetone. Antibodies specific for NFATc1 (BD Pharmingen), NFATc2, NFATc3, and NFATc4 (Santa Cruz Biotechnologies) were applied at dilutions of 1:100 at 4°C overnight, followed by appropriate Alexa555-labeled goat secondary antibodies (Molecular Probes) and visualized by fluorescent microscopy.

5.2.14 Statistical analysis

All experiments were replicated on 2-4 separate days. Results are presented as mean ± SEM. One-way ANOVA and Tukey's multiple comparisons tests were used to compare groups. A student's t-test was used to compare ATP levels at each time point examined (Prism; GraphPad Software). $p<0.05$ was considered statistically significant. No statistical differences were observed between vehicle controls (1-way ANOVA), so controls were combined. For calcium imaging, increases in intracellular calcium concentration were determined by calculating the average calcium concentration and standard deviation for individual cells before exposure to flow. A response was defined as a calcium concentration greater than the average concentration plus 4 standard deviations.

5.3 Results

5.3.1 Expression of P2 purinergic receptors by hMSCs

In Chapter 4 we found that oscillatory fluid flow induces human mesenchymal stem cell proliferation via a mechanism that involves an increase in intracellular calcium concentration and the activation of the MAP kinases ERK1/2 (513). Since the responses

to fluid flow of these marrow-derived progenitors are similar to responses observed in more mature osteoblastic cells, we hypothesized that flow-induced proliferation of hMSC may be mediated by the release of ATP and purinergic signaling.

As a first step in investigating this hypothesis, we examined the expression of purinergic receptors by hMSCs. Since previous studies indicated that the IP_3-mediated release of calcium from intracellular stores was important for fluid flow-induced proliferation, we focused on the expression of P2Y receptors, and the $P2X_7$ receptor given its role in bone cell mechanotransduction (10). We detected expression of the receptors $P2Y_2$, $P2Y_6$, $P2Y_{11}$ and $P2X_7$ by hMSCs via immunoblot (Figure 5-1). Specificity was confirmed by pre-incubation with control peptides which abolished immuno-reactivity. No immuno-reactivity was observed for the receptors $P2Y_1$, $P2Y_4$ or $P2Y_{12}$.

5.3.2 Fluid flow induces ATP release

We next examined the effect of oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 on ATP release from hMSCs using an ATP-dependent luciferin-luciferase reaction. Basal ATP levels in static hMSCs were on the order of 0.3±0.1nM (Figure 5-2A). Exposing hMSCs to oscillatory fluid flow induced a robust increase in ATP release. ATP levels peaked within one minute of the application of fluid flow at a concentration of 6.8±0.4nM and remained significantly elevated in conditioned media samples with prolonged exposure to fluid flow. While these results reflect the previously reported dynamics of ATP release from both an osteoblastic cell model and vascular endothelial cells in response to fluid flow, our results suggest that hMSCs release less ATP when compared with osteoblastic cells as Genetos *et al*. found that ATP levels peak at

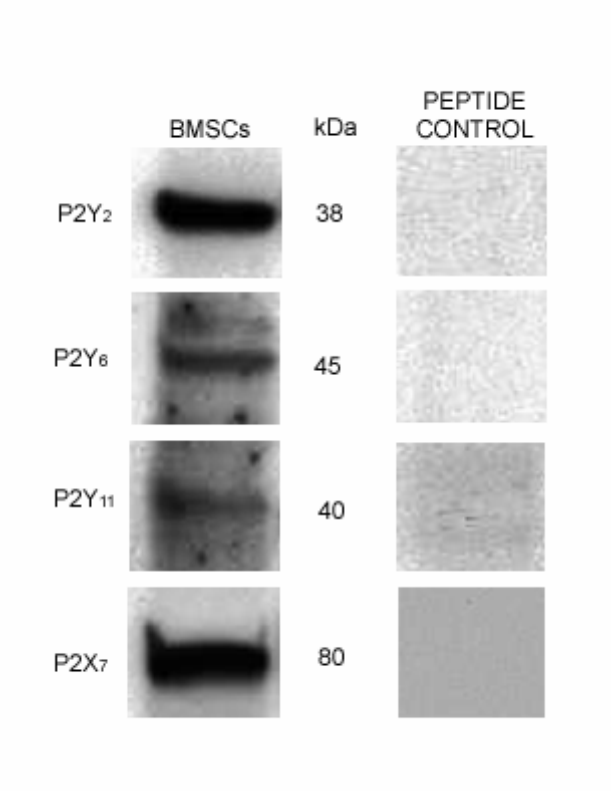

Figure 5-1. hMSCs express several P2 receptor subtypes. Cell lysates isolated from hMSCs were subjected to immunoblotting and expression of $P2Y_2$, $P2Y_6$, $P2Y_{11}$, and $P2X_7$ were routinely observed, while preincubation with peptide controls blocked immunoreactivity.

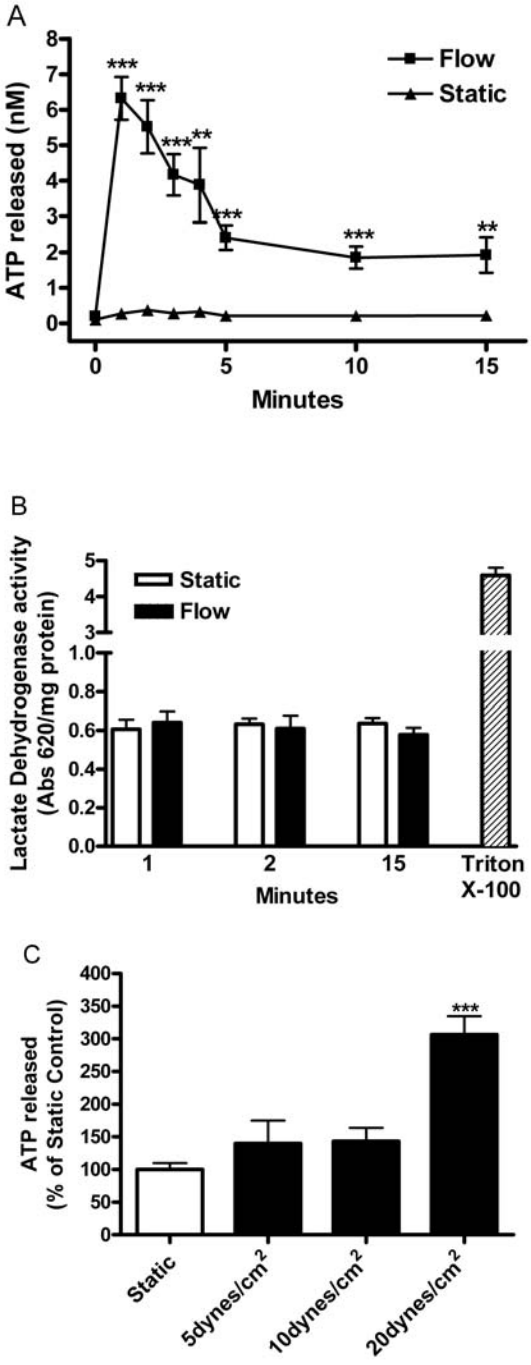

Figure 5-2. Oscillatory fluid flow induces ATP release from hMSCs. (A) hMSCs were exposed to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 at 1Hz or used as static controls and ATP levels in condition media samples examined at the indicated time points. (B) Lactate dehydrogenase activity levels were assessed in the same condition media samples to ensure the active release of ATP. Triton X-100 was used as a positive control. (C) hMSCs were exposed to fluid flow inducing shear stresses of 5, 10, or 20dynes/cm^2 for 5min and conditioned media samples collected and assessed for ATP release. ** $p<0.01$ compared to static controls; *** $p<0.001$ to static controls.

approximately 60nM when MC3T3-E1 cells are exposed to fluid flow (7,523). It is important to note that these measurements represent the concentration within the flow channel and are likely to be significantly higher at the cell surface after release.

To ensure that ATP release following fluid flow exposure was an active process rather than the result of plasma membrane disruption, we routinely measured lactate dehydrogenase (LDH) levels in conditioned media samples. If flow-induced ATP release results from membrane disruption, elevated levels of LDH would be detected in flow samples compared to static controls. We regularly observed similar LDH levels in conditioned media samples from cells exposed to fluid flow and static conditions (Figure 5-2B), suggesting that ATP release by hMSCs is an active response to oscillatory fluid flow.

Since we previously found fluid flow-induced proliferation and calcium signaling to be flow rate-dependent (513), we examined whether ATP release might also be dependent upon flow rate. Cells were exposed to flow rates inducing shear stresses of 5, 10, or 20dynes/cm^2 and conditioned media samples were collected. ATP levels in samples collected from cells exposed to fluid flow inducing a shear stress of 20dynes/cm^2 increased 206.5±28.5% over static controls (Figure 5-2C). Lower flow rates inducing shear stresses of 5 and 10dynes/cm^2 failed to significantly increase ATP release compared to static controls, exhibiting 39.8±35.5% and 43.4±20.4% increases over static controls, respectively.

5.3.3 ATP induces proliferation and contributes to fluid flow-induced proliferation

To determine whether the presence of extracellular ATP is sufficient to induce hMSC proliferation, hMCSs were treated with physiologic levels of ATP, ranging from 0 to

250μM (524), under static conditions. ATP treatment increased hMSC proliferation (Figure 5-3A). The percentage of BrdU incorporation in cells treated with 25, 100, and 250μM ATP for 20h were 240.0±14.1%, 239.1±20.4%, and 224.2±13.8% compared to untreated controls, respectively. A statistically significant increase in hMSC proliferation was not observed in cells treated with 5 μM ATP (139.9±14.1%).

To determine whether other nucleotide triphosphates might also induce hMSC proliferation, cells were treated with 100μM CTP, GTP, TTP or UTP (Figure 5-3B). hMSC proliferation was not affected by CTP, GTP, or UTP treatment (121.1±18.2%,129.0±15.9%, and 86.8±9.1%, respectively), while TTP treatment significantly decreased proliferation (48.9±6.1%). Other adenosine-based nucleotides, including ADP, AMP, and adenosine, also failed to significantly alter hMSC proliferation (137.8±17.0%, 122.9±12.7%, and 111.2±10.8%, respectively, Figure 5-3C) indicating that ATP is unique, among the nucleotides we examined, in its ablility to induce hMSC proliferation.

We next examined whether ATP release is directly responsible for fluid flow-induced proliferation of hMSCs, by exposing cells to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 in the presence or absence of apyrase (10U/ml), an enzyme which rapidly hydrolyzes extracellular nucleotides. As we have previously shown (Chapter 4 (513)), exposing hMSCs to fluid flow for 1hour induced a 122.7±20.1% increase in hMSC proliferation over unflowed, static controls (Figure 5-3D). Apyrase completely abolished the increase in hMSC proliferation in response to fluid flow (81.8±14.8%), without affecting proliferation in unflowed hMSCs (96.1±12.3%), suggesting that purinergic signaling is necessary for fluid flow to induce hMSC proliferation.

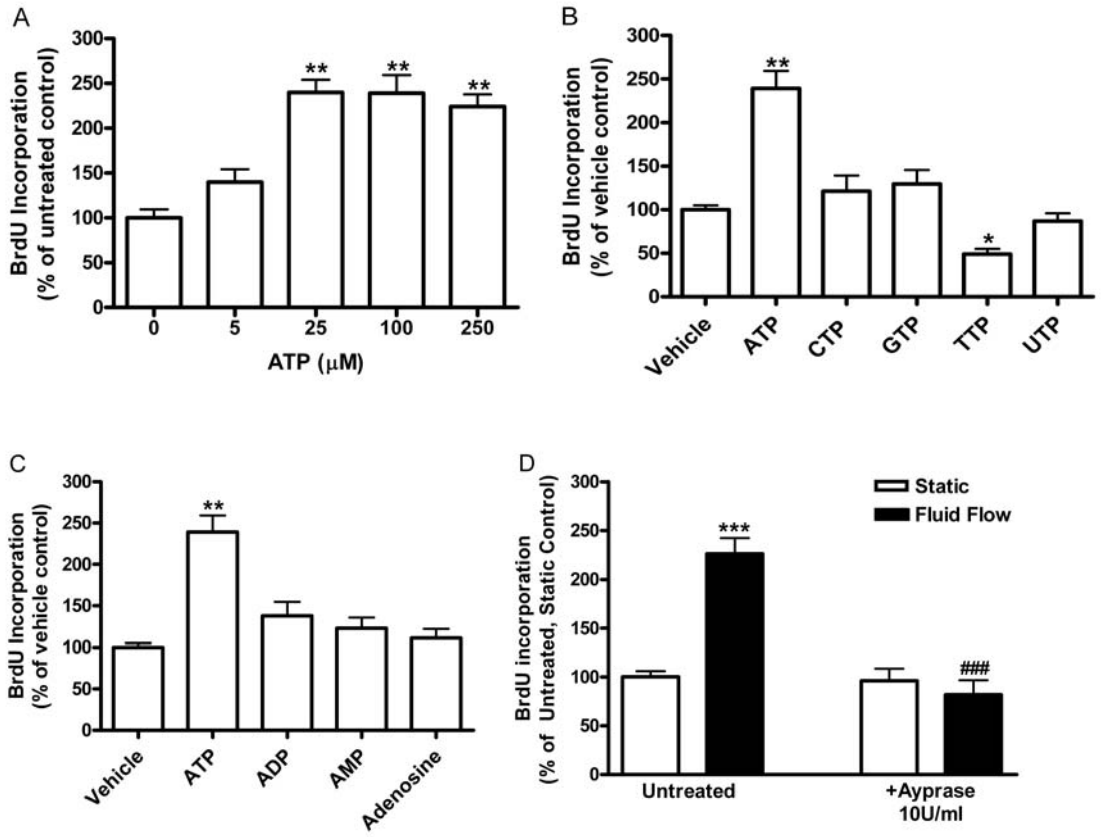

Figure 5-3. ATP induces hMSC proliferation. hMSCs were treated with 0-250μM ATP (A); 100μM CTP, GTP, TTP, UTP, or vehicle control (B); or, 100μM ADP, AMP, adenosine, or vehicle control (C). (D) hMSCs were left unflowed or exposed to oscillatory fluid flow for 1hour in the presence or absence of apyrase (10U/ml). After treatment, cells were returned to culture for 20 hours in flow media, and proliferating cells were then labeled 10μM 5-bromo-2-deoxyuridine (BrdU) for 1hour and quantified by FACS. *$p<0.05$ to vehicle control; ** $p<0.01$ compared to vehicle controls; *** $p<0.001$ to static controls; and ### $p<0.001$ to flow control.

5.3.4 ATP is released via vesicular exocytosis

To further clarify the mechanism by which oscillatory fluid flow induces hMSC proliferation, we examined the mechanism by which ATP is released. Since gap junction hemichannels have been implicated in the release of ATP from astrocytes (235), glioma cells (236), and osteocytes (525), we first examined whether hMSCs form functional hemichannels and whether antagonizing gap junction hemichannel activity with AGA (522,526) inhibits fluid flow-induced ATP release. Dye uptake studies utilizing the fluorescent tracer Lucifer Yellow (237,238) revealed that hMSCs are capable of forming functional hemichannels (Figure 5-4A). Treatment with 5mM EGTA, to lower extracellular calcium levels and open gap junction hemichannels (527,528), as well as exposure to oscillatory fluid flow induced Lucifer Yellow uptake by hMSCs, while few untreated cells took up Lucifer Yellow. Uptake of Rhodamine Dextran, indicative of endocytosis, was not observed in response to either EGTA or fluid flow treatment (data not shown). Importantly, pretreating hMSCs with 20μM AGA, which also blocked gap junction intercellular communication (Figure 5-4B), inhibited Lucifer Yellow uptake after EGTA or fluid flow exposure, implying that this process is mediated by gap junction hemichannels. However, we did not observe a significant difference between ATP release from AGA-treated hMSCs exposed to fluid flow compared to untreated controls (312.7±25.7% versus 254.4±35.8%, Figure 5-4C), suggesting that gap junction hemichannels do not contribute to the mechanism by which hMSCs release ATP in response to fluid flow.

As release of ATP from osteoblasts (7,240), endothelial cells (523), and epithelial cells (529) has been shown to occur via a vesicular mechanism, we examined whether

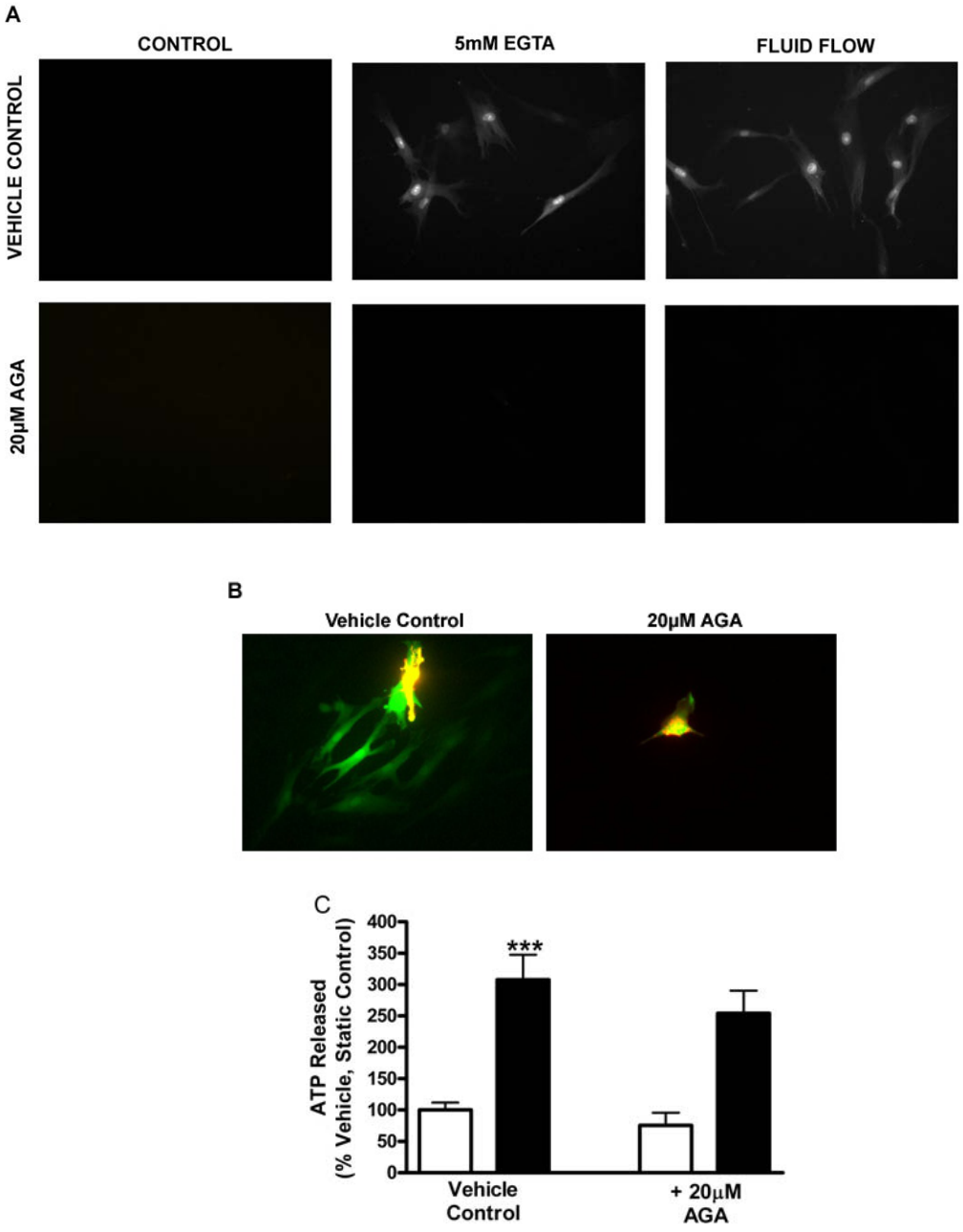

Figure 5-4. ATP is not released via gap junction hemichannels. (A) Hemichannel activity was examined in hMSCs via Lucifer Yellow dye uptake. Cells were left untreated, treated with 5mM EGTA, or exposed to oscillatory fluid flow in the presence or absence of 20μM AGA, and Lucifer Yellow uptake examined via fluorescent microscopy. (B) Gap junction intracellular communication was assessed by a double labeling technique in the presence of a vehicle control or 20μM AGA. Donor cells appear yellow, while cells communicating via gap junctions appear green. (C) hMSCs were left unflowed or exposed to fluid flow for 5min in the presence or absence of 20μM AGA to block hemichannel activity. *** $p<0.001$ compared to static controls.

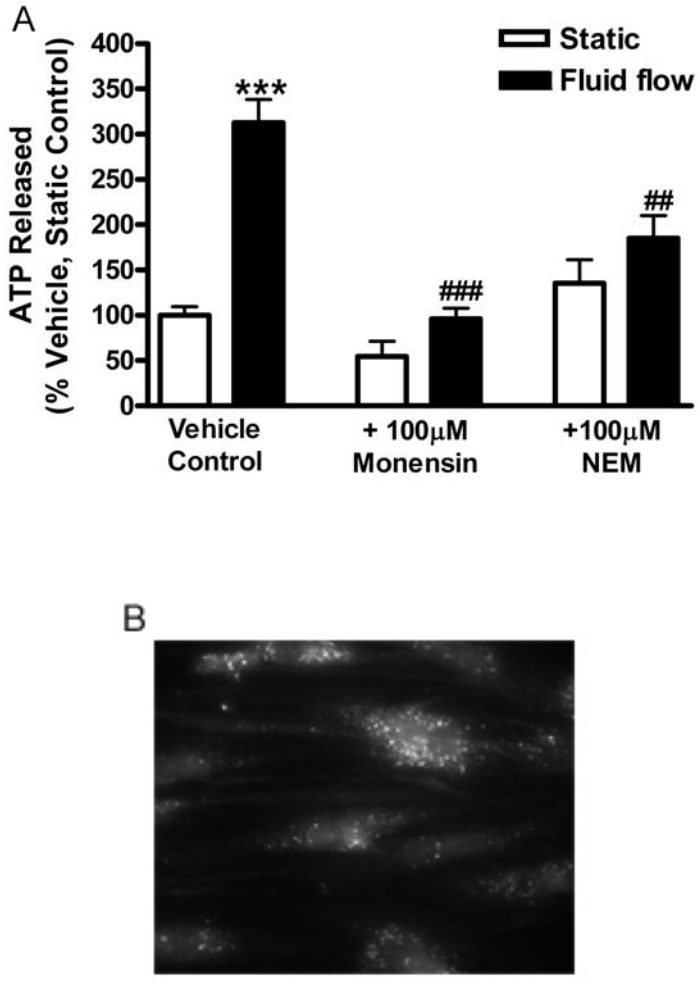

Figure 5-5. ATP is released via a vesicular mechanism. (A) hMSCs were left unflowed or exposed to fluid flow for 5min in the presence or absence of 100μM Monensin to inhibit vesicle formation or 100μM *N*-ethylmalemide (NEM) to prevent vesicle fusion with the plasma membrane and conditioned media samples collected and assessed for ATP levels. (B) hMSCs were stained with 25μM quinacrine to visualize intracellular ATP via fluorescent microscopy using a 40X objective. Note the intense punctuate staining indicative of ATP filled vesicles. *** $p<0.001$ compared to static controls. ## $p<0.01$ to flow control. ### $p<0.001$ to flow control.

this might be the case in hMSCs. Cells were pretreated with 100μM monensin, which prevents vesicle budding from the golgi apparatus (519,520), 100μM *N*-ethylmalemide (NEM), which prevents vesicle fusion with the cell membrane (521), or vehicle control and exposed to fluid flow. ATP release in response to fluid flow (312.7±25.7% of static controls) was significantly decreased by monensin and NEM treatment (96.1±11.7% and 185.1±24.9% of static control, respectively), suggesting that ATP is released from hMSCs in response to fluid flow via a vesicular mechanism (Figure 4B). Indeed, when we stained hMSCs with quinacrine (25μM), a fluorophore that binds ATP (530), we observed a diffuse, low-level fluorescence throughout the cytosol of hMSCs as well as intense, punctuate fluorescence indicative of ATP-filled vesicles (Figure 4C).

5.3.5 ATP release is responsible for the activation of calcium signaling pathways

Since we have found that fluid flow-induced proliferation by hMSCs depends upon intracellular Ca^{2+} signaling (513), and the release of ATP and subsequent activation of P2 receptors has been linked to increases in intracellular Ca^{2+} concentrations in osteoblastic cell models (11,240,505), we hypothesized that this may be the mechanism by which ATP release mediates flow-induced proliferation. To test this hypothesis, hMSCs were exposed to oscillatory fluid flow in the presence or absence of apyrase (10U/ml) and changes in intracellular Ca^{2+} concentration were assessed. In control samples, fluid flow stimulated robust increases in intracellular Ca^{2+} concentration 30s after the initiation of fluid flow with an amplitude of 184.9±12.1nM (Figure 5-6A, C). Apyrase treatment did not affect baseline intracellular calcium levels or the percentage of cells responding to fluid flow with an increase in intracellular calcium. However, the amplitude of the response to fluid flow was markedly attenuated, exhibiting an amplitude of 65.6±7.8nM

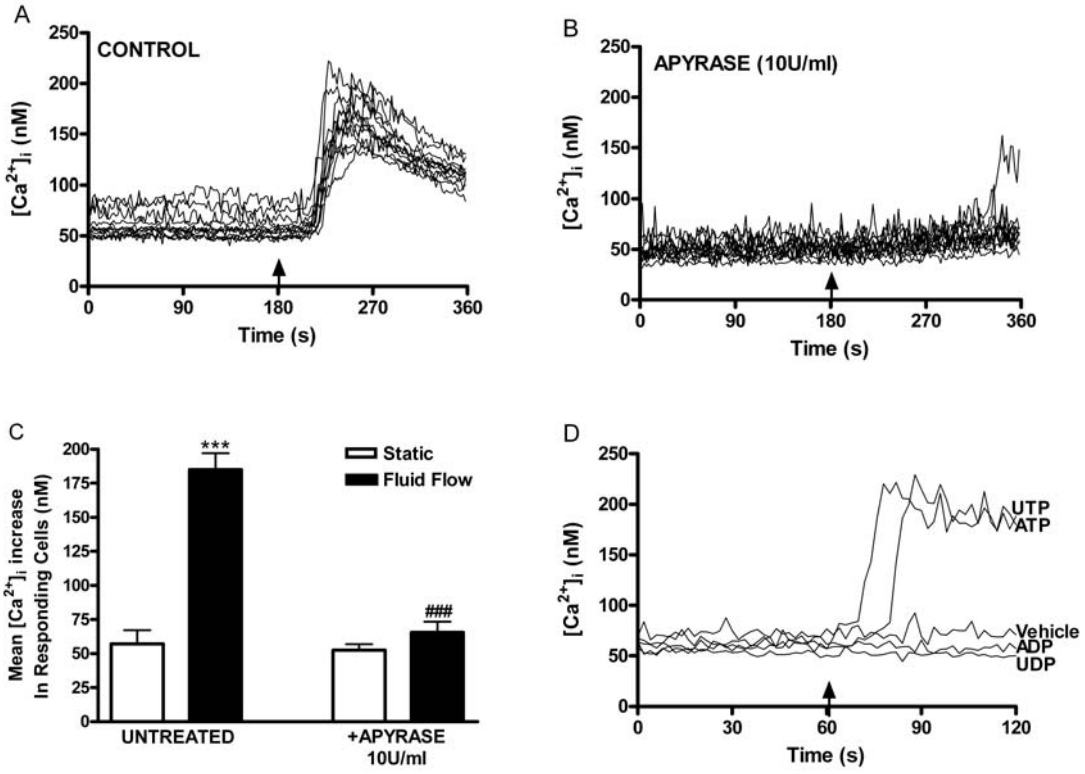

Figure 5-6. ATP release contributes to fluid flow-induced calcium signaling. hMSCs were exposed to fluid flow inducing a shear stress of 20dynes/cm^2 at 1 Hz in the presence (B) or absence (A) of apyrase (10U/ml). Each line represents the intracellular calcium concentration in a single cell, and the arrow represents the onset of flow. (C) Mean increase in intracellular calcium concentration in hMSCs responding to fluid flow. (D) Basal calcium levels were measured for 60sec and then hMSC were treated with the indicated nucleotide (25μM) or vehicle control (marked by arrow). *** $p<0.001$ compared with static control; ### $p<0.001$ compared to flow control.

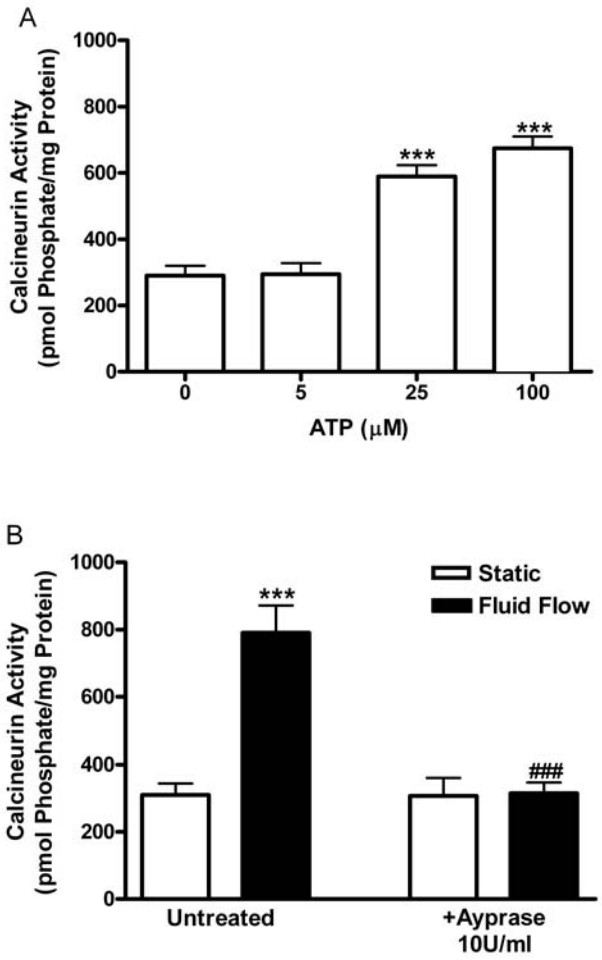

Figure 5-7. ATP release increases calcineurin phosphatase activity. hMSCs were treated with 0-100μM ATP for 5min (A), or were exposed to oscillatory fluid flow inducing a shear stress of 20dynes/cm^2 at 1Hz for 5min or used as time-matched static controls in the presence or absence of apyrase (10U/ml). Cellular lysates were incubated with a specific phosphopeptide, and liberated phosphate was quantified. *** $p< 0.001$ compared with untreated or static control; ### $p<0.001$ compared to flow control.

(Figure 5-6B, C). Treating hMSCs with 25μM ATP and UTP, but not ADP or UDP, induced similar increases in intracellular Ca^{2+} concentration (Figure 5-6D). These data suggest that while ATP release and purinergic signaling are major contributors to the effect of fluid flow on intracellular calcium concentration, other mechanisms are also activated by fluid flow.

Calcineurin, a calcium-responsive phosphatase, has been linked to the regulation of cellular proliferation via the regulation of cyclin D accumulation (498) and represents a target for fluid flow-induced Ca^{2+} transients. We examined whether ATP treatment under static conditions, as well as degrading extracellular nucleotides during fluid flow exposure, alters calcineurin activity levels. Treating hMSCs with 25μM and 100μM ATP increased calcineurin phosphatase activity from 290.0±29.7pmol phosphate liberated/mg protein in untreated samples to 589.3±34.1 and 673.3±35.9pmol phosphate liberated/mg protein, respectively (Figure 5-7A). Treatment with 5μM ATP was not sufficient to increase calcineurin activity (293.9±33.2 pmol phosphate liberated/mg protein). As we found previously (513), calcineurin activity levels increased significantly from 309.6±33.8pmol phosphate liberated/mg protein in unflowed cells to 790.3±81.6pmol phosphate liberated/mg protein in cells exposed to fluid flow (Figure 5-7B). In the presence of apyrase, there was no significant change in calcineurin activity between unflowed cells and those exposed to fluid flow (306.7±53.5 versus 314.0±31.7pmol phosphate liberated/mg protein), indicating that extracellular ATP and the activation of purinergic signaling is necessary for fluid flow to activate calcineurin.

In light of these findings, we hypothesized that the activation of purinergic signaling by fluid flow in hMSCs induces the nuclear translocation of NFAT, a target of calcineurin

(478). By immunofluorescence, we confirmed that hMSCs express NFATc1, NFATc3, and NFATc4 which were localized to cytosolic or perinuclear regions within the cell (Figure 5-8A). Expression of NFATc2 was not detected (data not shown). In response to fluid flow exposure (30min), we observed the nuclear translocation of NFATc1, while NFATc3 and NFATc4 appear to be retained in the cytosol. To ensure that the nuclear translocation of NFATc1 was mediated by purinergic signaling, we treated cells with ATP or exposed hMSCs to fluid flow in the presence of apyrase. In hMSCs treated for 30 min with 25μM or 100μM ATP, doses that induce hMSC proliferation (Figure 5-3A), NFATc1 translocated from the cytosol to the nucleus (Figure 5-8B). Intriguingly, treatment with 5μM ATP, a concentration not sufficient to induce hMSC proliferation, was also unable to induce NFATc1 translocation. Finally, we found that treating hMSCs with apyrase prior to and during fluid flow exposure prevented the nuclear translocation of NFATc1 (Figure 5-8C). In untreated controls, NFATc1 staining was primarily localized to the cell nucleus after 30min of fluid flow. In cells treated with apyrase, which abolished the increase in intracellular calcium concentration and the activation of calcineurin in response to fluid flow, NFATc1 remained in the cytosol after fluid flow exposure. These data indicate that purinergic signaling is an important mediator of the intracellular calcium signaling pathways necessary for fluid flow to induce hMSC proliferation.

5.3.6 Degrading extracellular nucleotides does not inhibit ERK1/2 phosphorylation

Since we found that MAP kinase signaling contributes to the effect of fluid flow on hMSC proliferation (513), we examined whether extracellular nucleotides contribute to the activation of MAP kinase signaling in response to fluid flow. Treating hMSCs with 25μM ATP, a concentration sufficient to induce hMSC proliferation (Figure 5-3A), increased

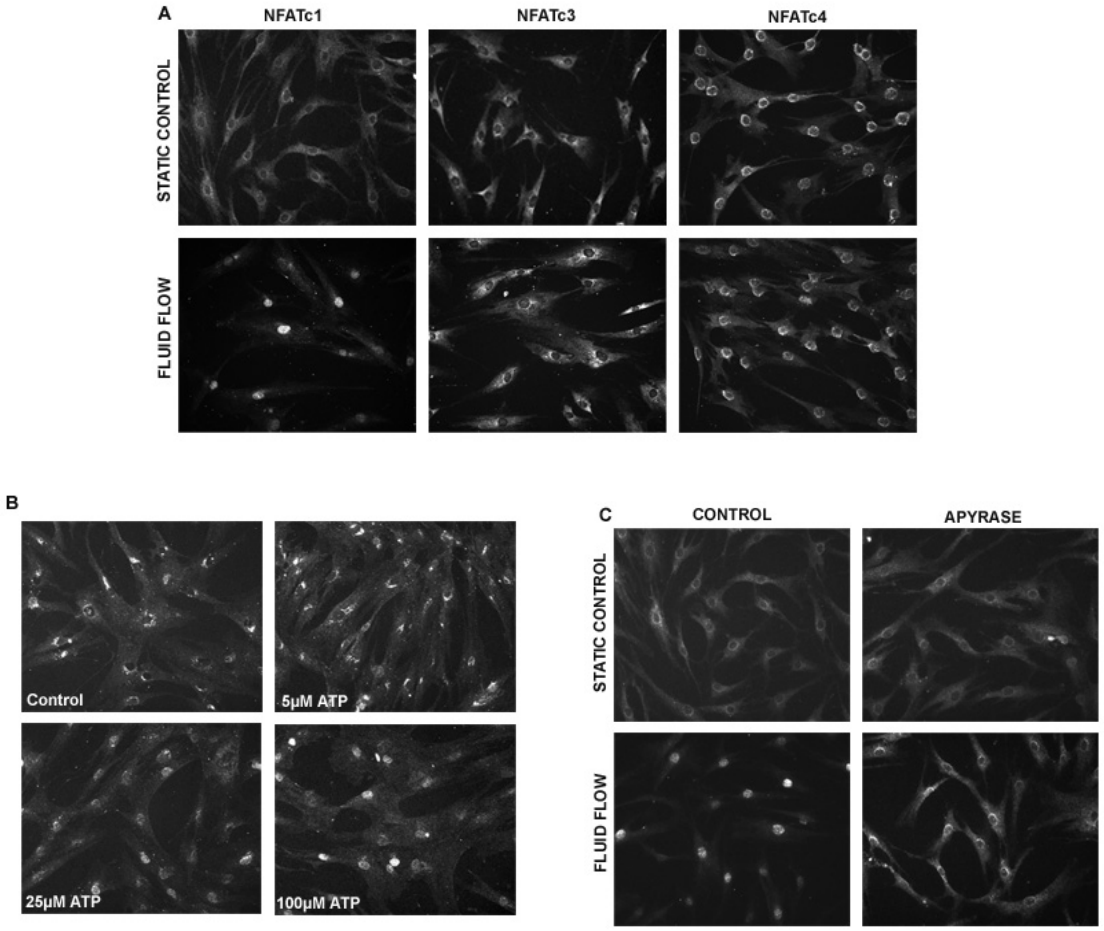

Figure 5-8. Fluid flow and ATP induced the nuclear localization of NFATc1. hMSCs were exposed to fluid flow (A,C) or treated with ATP for 30min (B) and the cellular localization of NFAT isoforms examined by immunofluorescence. Expression of NFATc1, NFATc3 and NFATc4 were detected in hMSCs. NFATc1 translocated to the nucleus in response to fluid flow exposure or treatment with 25μM or 100μM ATP. Apyrase treatment (10U/ml) blocked the nuclear translocation of NFATc1 in response to fluid flow (C).

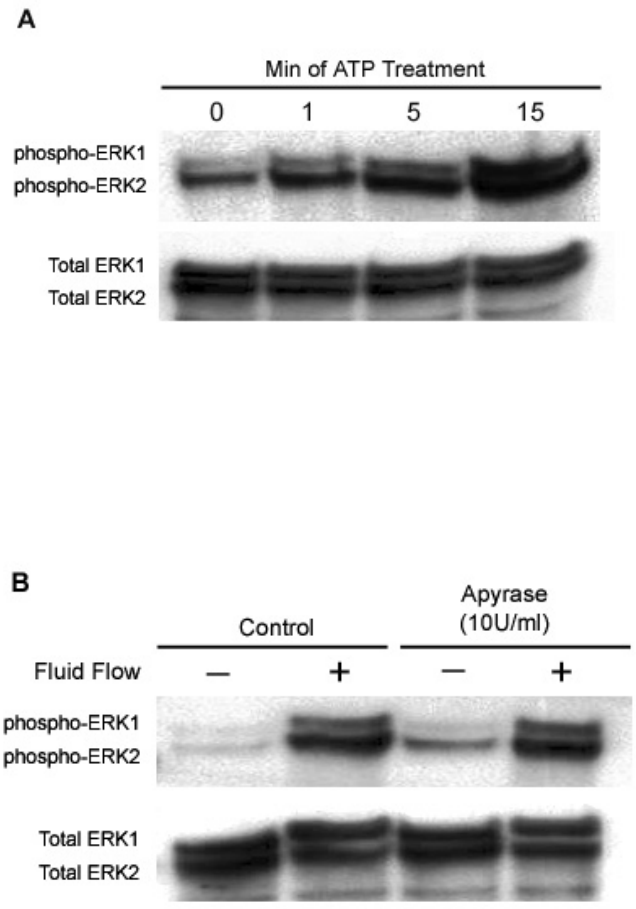

Figure 5-9. Degrading ATP does not inhibit ERK1/2 phosphorylation. (A) hMSCs were treated with 25μM ATP for 0, 1, 5, or 15min and ERK1/2 phosphorylation levels assessed via immunoblotting. (B) hMSCs were exposed to fluid flow inducing a shear stress of 20dynes/cm^2 or used as static controls for 15min in the presence or absence of apyrase (10U/ml).

ERK1/2 phosphorylation levels 1, 5, and 15min after treatment (Figure 5-9A), indicating that extracellular nucleotides are sufficient to activate MAP kinase signaling. However, degrading extracellular nucleotides with apyrase (10U/ml) did not alter ERK1/2 phosphorylation compared to flow controls (Figure 5-9B). These data suggest that other, non-purinergic signaling cascades activated by fluid flow induce MAP kinase signaling.

5.4 Discussion

The anabolic effect of mechanical loads on skeletal homeostasis is likely to require the coordinated efforts of multiple bone cell types including osteocytes, osteoblasts, and mesenchymal stem cells. To date, most studies have focused on the effects of mechanical signals on the behavior and activity of mature bone cells, but accumulating evidence suggests that, like osteoblasts and osteocytes, mesenchymal stem cells are mechanosensitive (450-452,513). These cells, which have the potential to differentiate to cells of the osteoblastic, adipocytic and chondrogenic lineages (284,472), could contribute to the anabolic response of bone to mechanical load through their proliferation and differentiation to osteoblastic cells. Additionally, a complete understanding of the effects of biophysical signals on hMSC behavior could be utilized in tissue engineering protocols.

We have recently shown that exposing hMSCs to oscillatory fluid flow induces cellular proliferation via a mechanism that involves the activation of MAP kinase and calcium signaling cascades (Chapter 4)(513). However, the mechanism by which these signaling cascades are activated has not been identified. Since several studies have indicated that extracellular nucleotides contribute to bone cell mechanotransduction

(7,10,11,506), we hypothesized that the effect of fluid flow on hMSC proliferation is mediated by the release of ATP.

We found that hMSCs express a number of purinergic receptors, including the P2Y2 and P2X7 receptors, that have already been implicated in bone cell mechanotransduction (10,11). Further, in response to fluid flow exposure, hMSCs rapidly released ATP into the extracellular environment, and our data suggest that the release of this nucleotide is necessary for fluid flow to induce hMSC proliferation. When we degraded extracellular nucleotides with the enzyme apyrase, increases in cellular proliferation after fluid flow exposure were not observed. While it is possible that hMSCs release other nucleotides in response to fluid flow that could be degraded by apyrase, increasing the extracellular concentration of these nucleotides did not affect hMSC proliferation. In static experiments we found that only ATP treatment was able to significantly increase hMSC proliferation.

Interestingly, the release of ATP in response to fluid flow was dependent upon the flow rate. ATP levels in conditioned media samples collected from cells exposed to fluid flow inducing a shear stress of 5 or 10dynes/cm^2 were not significantly increased over static controls, while a flow rate inducing a shear stress or 20dynes/cm^2 induced a robust and significant increase in ATP release. These data may explain the flow rate-dependent effects of fluid flow on intracellular calcium signaling and cellular proliferation that we observed previously (Chapter 4)(513). However, it is not clear whether this observation is related to an increase in fluid shear stress or increased chemotransport due to higher flow rates. Previous studies from our laboratory have suggested that chemotransport contributes to the effects of fluid flow on bone cells (6), but conflicting reports suggest that fluid shear stress is the determining stimulus (170,268). The only study to examine

the effect of fluid shear stresses on mesenchymal stem cell proliferation did not report a shear dependent effect on proliferation (515). However, the different fluid flow regimes, cell models, and experimental endpoints utilized in these reports suggest that further study is necessary.

When taken together with previous studies, our data suggest the mechanism by which ATP is released from bone cells is dependent upon the stage of differentiation. Two studies have suggested that osteoblastic cells release ATP through a vesicular mechanism (7,240) but release from terminally differentiated osteocytic cells occurs through the opening of hemichannels (239), unopposed gap junction channels found on the surface of cells that allow the passage of small molecules to the extracellular environment. Recent studies from our laboratory and others (238,239) suggest that these channels are recruited to the cell surface and activated by fluid flow. In this study, we found that hMSCs form functional hemichannels that are activated by both fluid flow exposure and by lowering extracellular calcium concentrations. However, hemichannels do not appear to represent the major mechanism by which hMSCs release ATP. Treating cells with AGA, which inhibited dye uptake through gap junction hemichannels, did not significantly affect ATP release. Rather our data suggest that fluid flow stimulates ATP release from hMSCs via a vesicular mechanism. Staining cells with quinacrine, an anti-malaria drug with high affinity for ATP (530), revealed a punctuate staining pattern suggesting the presence of ATP-filled vesicles similar to those identified in osteoblastic cells (7,240) and endothelial cells (523). Further, inhibiting vesicular trafficking with monensin, (519,520) and *N*-ethylmalemide (521), significantly decreased flow-induced ATP release. One possible explanation for this shift in the mechanism by which ATP is released may be the

expression level of connexin proteins. The expression of connexin 43, the predominate connexin expressed by bone cells reportedly increases with osteoblastic differentiation (531), possibly allowing gap junction hemichannels to increase in prevalence and increasing their functional importance in bone cell mechanotransduction.

Activation of purinergic signaling contributed to intracellular calcium signaling in hMSCs. While a similar number of cells responded to fluid flow with an increase in intracellular calcium concentration, apyrase markedly decreased the amplitude of fluid flow-induced calcium transients compared to those observed in untreated controls. The amplitude and duration of calcium signals have been reported to provide specificity among cellular responses (475,476), and indeed, we found that attenuating the amplitude of flow-induced calcium signals with apyrase prevented the activation of the downstream targets, calcineurin and NFAT.

Recently Winslow *et al* (260) reported that the expression of a constitutively nuclear NFATc1, induces a high bone mass phenotype through an increase in osteoblast proliferation. However, it was unclear in this study what signal would induce the activation of this signaling cascade. Our study provides the first evidence that NFAT is regulated by mechanical stimulation in bone cells and suggests that it is a mediator of this response. We found that hMSCs express NFATc1, NFATc3 and NFATc4, but only the nuclear localization of NFATc1 was induced by oscillatory fluid flow. While the observation that only a single NFAT isoform is activated by fluid flow is intriguing, it is not unique to hMSCs. Endothelial cells also express multiple NFAT isoforms, but stimuli like VEGF induce the nuclear translocation of only a single isoform (532). At present, it is not clear whether the nuclear translocation of NFATc1 contributes to the

effect of fluid flow on hMSC proliferation by directly altering the expression of cell cycle-related genes or by enhancing the expression of an autocrine factor. NFAT has been shown previously to control the expression of cyclin-dependent kinase 4 (533), but regulation of cell cycle progression by NFATc1 in 3T3-L1 cells requires an autocrine intermediate (534). Further study will be necessary to discern the exact role NFATc1 plays in hMSC proliferation.

In Chapter 4, we found that MAP kinase signaling also contributes to the effect of fluid flow on hMSC proliferation (513), and we speculated that this signaling cascade would also be activated via purinergic signaling in response to fluid flow. Here, we found that while ATP was sufficient to induce ERK1/2 phosphorylation, degrading extracellular nucleotides did not abolish ERK1/2 phosphorylation in response to fluid flow. These data suggest that other non-purinergic signaling cascades must also induce MAP kinase signaling in response to fluid flow. Possibilities include integrin signaling (228,535) or calcium conducting channels (493).

In summary, these studies provide evidence that ATP and purinergic signaling is an important mediator of hMSC proliferation. Like osteoblasts and osteocytes, hMSCs released ATP in response to fluid flow and this autocrine/paracrine factor was necessary for fluid flow to stimulate hMSC proliferation through the activation of intracellular calcium signaling pathways. These data support our hypothesis that fluid flow regulates not only osteoblastic and osteocytic cell behavior but also that of hMSCs and imply that mechanical signals could be used in the development of novel therapeutic techniques or tissue engineering protocols.

CHAPTER 6

CHEMOTRANSPORT CONTRIBUTES TO THE EFFECT OF FLUID FLOW ON CELLULAR PROLIFERATION

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Mechanical loads produce a diverse set of biophysical signals that may regulate bone cell activity, but accumulating evidence suggests that interstitial fluid flow is the primary signal that bone cells perceive. Since we previously demonstrated that fluid flow increases human mesenchymal stem cell proliferation, we investigated the contribution of fluid shear stress and chemotransport, two biophysical stimuli induced by interstitial fluid flow. Decreases in chemotransport at a constant peak shear stress were associated with decreases in fluid flow-induced hMSC proliferation, while variations in peak fluid shear stress had no significant effect. Modulation of hMSC proliferation by chemotransport may be attributed to changes in the release of ATP and intracellular calcium signaling. We found that if levels of chemotransport are decreased while maintaining a constant peak fluid shear stress, hMSCs release less ATP into the extracellular environment. Moreover, as chemotransport decreased, fewer cells responded to fluid flow with an increase in intracellular calcium concentration. These data suggest that the clearance of cellular metabolites or sufficient nutrient supply is a prerequisite for hMSCs to respond to interstitial fluid flow.

6.1 Introduction

Regulation of bone cell behavior by mechanical signals is widely accepted and accumulating evidence suggests that of the many mechanical stimuli that bone cells might respond to, interstitial fluid flow is the most potent (205,206). Deformation of skeletal tissue in response to load induces the pressurization of interstitial fluid and its movement along pressure gradients (4). *In vitro* studies reveal that the effects of fluid flow stimulation on bone cell behavior reflect the effects of mechanical loads on bone cells. Short-term exposure (seconds to minutes) of bone cells to fluid flow triggers the

release of ATP (7,536), an increase in intracellular calcium (210,254,258) and the activation of intracellular signaling cascades such as MAP kinase (210,264,265), PKA (266), and PKC (267). Longer term exposure (minutes to hours) to fluid flow induces the production and release of a number of paracrine factors including nitric oxide (9,170,215) and prostaglandin E_2 (7,238,518), as well as alteration in gene expression. Examples of fluid flow responsive genes include cox-2 (245), c-fos (258), and several extracellular matrix proteins (210,211). Inhibition of many of these responses abolishes the anabolic response of bone to mechanical loads *in vivo* (232,250,251). While the mechanisms by which bone cells respond to interstitial fluid flow are being elucidated, the stimulus that bone cells perceive remains unclear.

Interstitial fluid flow in and of itself produces a number of stimuli which bone cells may perceive, including streaming potentials, fluid shear stress, and chemotransport. Detection of streaming potential or electric potentials generated by the movement of ions across the charged cell membrane by bone cells has largely been disproven (537). However, reports on the contributions of chemotransport and fluid shear stress to the responses of bone cells to fluid flow are contradictory. Independent studies by Haut Donahue *et al*. (6) and Allen *et al*. (214) indicate that chemotransport, the clearance of cellular metabolites and replacement of nutrient levels, is necessary for flow-induced responses, as the mobilization of intracellular calcium and release of prostaglandin were significantly dimished or abolished in media devoid of nutrients or that has a low serum concentration. However, data from McAllister and Frangos (215), as well as Bakker *et al*. (170), suggest that shear stress rather than chemotransport is the determining stimulus in the response of bone cells to fluid flow. Complicating these discrepancies are the

differences in fluid flow regimes (oscillatory, steady, or pulsatile) and variety of bone cell models utilized in these studies. Thus, further examination is necessary to clarify the effects of chemotransport and fluid shear stress.

Recently, we and others have found that human mesenchymal stem cells (hMSCs) respond to oscillatory fluid flow in a manner similar to osteoblasts and osteocytes (452,513). *In vivo*, this cell population has the potential to differentiate along the osteoblastic, adipocytic, and chondrogenic lineages (284,472), and could be exposed to fluid flow resulting from the generation of intramedullary pressure associated with mechanical loads (457,458) or as they migrate to sites of bone formation through haversian systems where flow is predicted to induce shear stress similar to those hypothesized for the lacunar-canilicular network (5,209,538,539). Interestingly, many of the responses we previously observed in hMSCs, including the mobilization of intracellular calcium, the release of ATP, and cellular proliferation are flow rate-dependent (513,536). However, variations in the flow rates used in these studies to induce fluid shear stress ranging from 5 to 20dynes/cm^2 precluded the differentiation between the effects of peak shear stress and chemotransport. The goal of this study was to investigate the contributions of chemotransport and fluid shear stress on oscillatory fluid flow-induced hMSC proliferation and the signaling cascades leading to this response. We demonstrate that chemotransport is a major contributor to the effects of fluid flow on hMSC behavior as decreases in flow rate while maintaining a constant fluid shear stress are associated with alterations in the proliferation response of hMSCs to fluid flow, likely due to alterations in the release of ATP and intracellular calcium signaling.

6.2 Materials and Methods

6.2.1 Cell culture

Human mesenchymal stem cells from an 18 year old, male donor were obtained from a commercial source (Cambrex Biosciences) and cultured in DMEM-low glucose (Invitrogen) supplemented with 10% FBS (Hyclone), 1% penicillin/streptomyocin, and 2mM L-glutamine according to standard techniques. At 85% confluence, hMSCs were subcultured and seeded at a density of $5x10^3$cells/cm^2 to either 76 x 26 x 1.6mm quartz slides for calcium imaging experiments or to 75 x 38 x 1mm glass slides for cellular proliferation and ATP release experiments. Forty-eight hours after seeding when cells had obtained 80% confluency, culture medium was replaced with standard flow medium (DMEM-low glucose containing 0.5% FBS), and cells were cultured for an additional 24h before exposure to fluid flow. hMSCs from passage 3-7 were utilized in these studies.

6.2.2 Induction and assessment of osteoblastic differentiation

The osteoblastic differentiation of hMSCs was induced by treating 70% confluent cultures with growth medium supplemented with 10nM dexamethasone, 10mM β-glycerol phosphate, and 50μg/ml ascorbic acid phosphate. After 12 days of differentiation, osteogenic cells were collected by sequential treatment with 1mg/ml collagenase and 0.25% trypsin/1mM EDTA and seeded to quartz slides as above or to 6well plates. On the day of the experiment, cells grown in 6well plates were collected in 0.1% Triton X-100, 10mM Tris pH 8, 1mM EDTA and a protease inhibitor cocktail (Calbiochem), and levels of alkaline phosphatase activity measured using a colorimetric assay to assess osteoblastic differentiation. In an alkaline solution (pH 8), alkaline phosphatase in cell lysates converted p-nitrophenolphosphate (3mg/ml) to p-nitrophenol,

the absorbance of which was measured at 405nm (MRX; Dynex Technologies) and compared to known p-nitrophenol standards. Results were normalized to total protein concentration determined using the bicinchoninic acid method (Pierce).

6.2.3 Application of fluid flow

hMSCs were exposed to oscillatory fluid flow as described previously (6,513,517). Slides containing cell monolayers were positioned in parallel plate flow chambers modified from those described by Frangos *et al*. (482) and connected to a servopneumatic materials testing device (EnduraTec), oscillating at 1Hz, via glass Hamilton syringes and rigid wall tubing. For calcium imaging experiments, a chamber with a fluid volume of 34 x 10 x 0.28mm was used, whereas a larger chamber with a fluid volume of 75 x 34 x 0.28mm was used for cellular proliferation and ATP release experiments. The equation

$$\tau=6Q\mu/bh^2 \quad \text{(Equation 1)}$$

was employed to determine the relationship between flow rate and fluid shear stress. τ is the wall shear stress (dyn/cm^2), Q is the flow rate (cm^3/s) monitored in real-time using an ultrasonic flowmeter (Transonic Systems), and μ is the viscosity of the flow medium (dyn·s·cm^{-2}), while b and h are the width and height of the channel, respectively (482). hMSCs were exposed to steady fluid flow using a Harvard syringe pump. Cells were exposed to fluid flow in standard flow media in the presence or absence of neutral dextran or Hank's balanced salt solution (HBSS) in the presence or absence of 0.5% FBS.

6.2.4 Viscosity Measurements

A Brookfield Synchro-lectric viscometer, calibrated with ultrapure water, was used to assess the viscosity of flow media. Standard flow media, HBSS, and HBSS containing 0.5% FBS had a viscosity of 1.0cP (1.0centiPoise (cP)=0.01dyn·s/cm^2). Addition of 1, 2,

or 3% (w/v) of neutral dextran (Sigma, MW 500,000) increased the viscosity to 1.5, 2.4, and 3.8cP, respectively.

6.2.5 Calcium imaging

Intracellular calcium concentration was determined using the ratiometric calcium indicator Fura-2 (Molecular Probes) as we have described previously (210,513). hMSCs were incubated in a 10μM Fura-2AM solution for 30min at 37ºC and then positioned on a parallel plate flow chamber on an inverted fluorescent microscope (Nikon). Fura-2 exhibits a shift in absorption when bound to Ca^{2+}, such that the emission intensity following ultraviolet illumination increases with $[Ca^{2+}]_i$ at 340 nm and decreases with $[Ca^{2+}]_i$ at 380 nm. Metafluor image analysis software (Universal Imaging) was used to compare consecutive images at 340 and 380nm recorded once every 2s to a calibration curve provided by the manufacturer. Basal $[Ca^{2+}]_i$ levels were sampled for 3min to serve as a static control followed by 3min of oscillatory fluid flow.

6.2.6 Cellular proliferation assays

hMSC proliferation was assessed using a FITC 5-bromo-2'-deoxyuridine (BrdU) flow kit (BD Pharmigen). Cells were exposed to oscillatory fluid flow for 1h or used as static controls and then incubated for 20h in fresh flow medium at 37°C with 5% CO_2. Proliferating cells were then labeled for 60min with 10μM BrdU. Cells were subsequently washed with PBS, collected in a 0.25% trypsin/1mM EDTA solution, fixed in 2% paraformaldehyde, and the percentages of proliferating cells were quantified using fluorescence-activated cell sorting (FACS; FACScan, BD Pharmigen).

6.2.7 Luciferin-luciferase determination of ATP concentration

Following 5min of fluid flow exposure, conditioned media samples were collected and immediately stored at -80°C until the time of analysis. ATP concentrations in conditioned media samples were determined using a commercially available ATP bioluminescence determination kit (ATP bioluminescence assay kit HS II, Roche). ATP in each conditioned media sample was used by luciferase as a cofactor to convert D-luciferin in a luciferin-luciferase assay buffer into oxyluciferin and light. The luminescence from each reaction was measured with a Monolight 3010 luminometer (BD Pharmigen) and compared to a standard curve created by serially diluting an ATP standard. Duplicate measurements were taken from each conditioned media sample, and control experiments were performed with neutral dextran to ensure its presence had no detrimental effect on the reaction. Results were normalized to cellular protein concentration using the bicinchoninic acid method (Pierce).

6.2.8 Lactate dehydrogenase measurements

The presence of lactate dehydrogenase (LDH), a cytosolic enzyme quickly released from damaged cells, in conditioned media samples was used to assess plasma membrane integrity. LDH levels were assessed using a Cytotox96 Nonradioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions. In the presence of LDH, lactate is oxidized to pyruvate, producing NADH that is used as a cofactor in the conversion of iodonitrotetrazolium into a red formazan product in the presence of diaphorase. Absorbances at 490nm, which are proportional to the amount of LDH present in conditioned media samples, were compared between static and fluid flow

samples. Cell lysates collected in 0.1% Triton X-100, 10mM Tris pH 8, 1mM EDTA were used as a positive control for LDH activity.

6.2.9 Statistical analysis

All experiments were replicated on at least 3 separate days. Increases in $[Ca^{2+}]_i$ during the static period or in response to fluid flow were determined by calculating the average $[Ca^{2+}]_i$ and standard deviation for individual cells before exposure to flow. A calcium concentration greater than the average $[Ca^{2+}]_i$ plus 4 standard deviations in response to flow or during the static period was defined as a response. All data are expressed as means ± SEM. One-way ANOVA and Tukey's multiple comparisons tests were used to compare groups (Prism; GraphPad Software). $p<0.05$ was considered statistically significant.

6.3 Results

6.3.1 Decreasing chemotransport alters calcium signaling

As an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) has widely been used as an indicator of the responsiveness of bone cells to fluid flow (6,214,254,517), we first examined the effect of altering chemotransport while maintaining a peak fluid shear stress of 20dynes/cm^2 on flow-induced calcium transients. A small fraction of hMSCs (1.1±0.5%) exhibited spontaneous increases in $[Ca^{2+}]_i$ during the static period and these response were not altered by increasing the viscosity of the flow media with 1% or 2% neutral dextran (Figure 6-1A,B). In response to a flow rate of 18ml/min inducing a peak shear stress of 20dynes/cm^2, 94.0±1.6% of hMSCs exhibited an increase in $[Ca^{2+}]_i$ with a mean amplitude of 212.7±15.5nM (Figure 6-1C). When the flow rate (chemotransport) was decreased to 11.5ml/min and 7.2ml/min while maintaining a peak shear stress of 20dynes/cm^2, we observed a significant decrease in both the percentage of cells

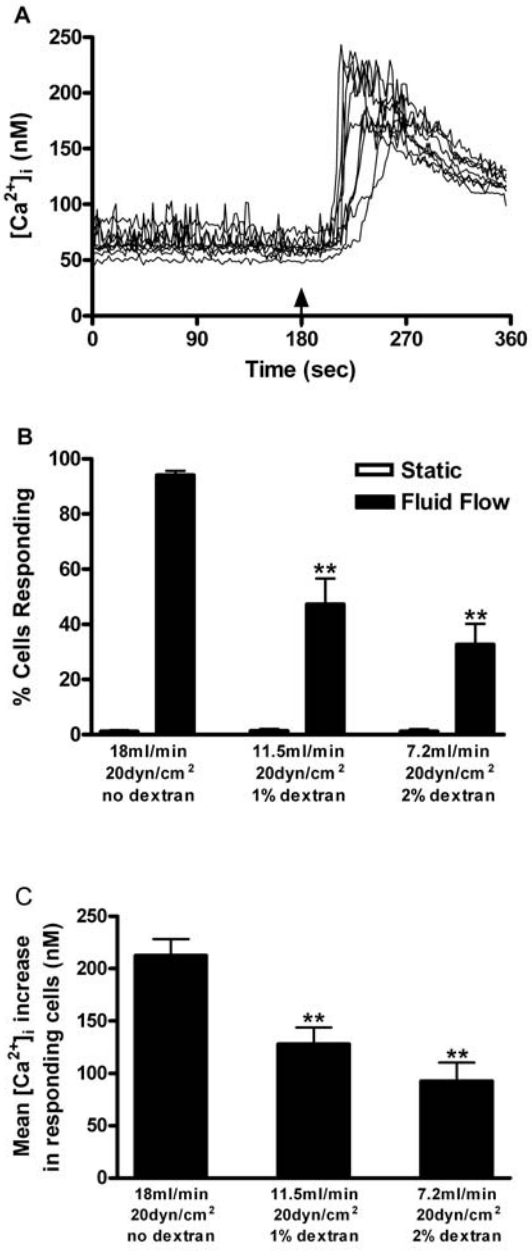

Figure 6-1. Effect of chemotransport on calcium signaling. (A) A typical calcium trace from hMSCs exposed to oscillatory fluid flow with a flow rate of 18ml/min and a peak shear stress of 20dyn/cm^2. Each line represents the $[Ca^{2+}]_i$ in a single cells, while the arrow denotes the initiation of fluid flow. In subsequent experiments, peak fluid shear stress was held constant at 20dyn/cm^2 by adding neutral dextran to the flow media, while chemotransport was varied by decreasing the flow rate, and the percentage of cells responding (B) and the mean increase in $[Ca^{2+}]_i$ in cells responding to fluid flow (C) were quantified. Open bars indicate the percentage of cell responding under static conditions, while filled bars represent the percentage of cells responding to fluid flow. ** $p<0.01$ compared to from 18ml/min, 20dyn/cm^2, no dextran.

responding (47.3±9.3% and 32.9±7.6%, respectively) as well as the amplitude of the response (128.1±15.8nM and 92.6±17.6nM, respectively) suggesting that chemotransport is an important stimulus in the responsiveness of hMSCs to oscillatory fluid flow.

6.3.2 Removing chemotransport greatly diminishes calcium signaling

To confirm the effects of chemotransport on hMSCs, we exposed cells to fluid flow in Hank's buffered salt solultion (HBSS), which is devoid of nutrients, at a flow rate of 18ml/min and peak shear stress of 20dynes/cm^2. As indicated above, in standard flow media 94.0±1.6% of hMSCs responded to fluid flow with a 212.7±15.5nM increase in $[Ca^{2+}]_i$. In HBSS, only 16.6±5.0% of cells responded to fluid flow with an increase in $[Ca^{2+}]_i$ with a mean amplitude of 54.3±6.8nM (Figure 6-2A,B). Adding 0.5% serum to HBSS rescued the ability of hMSCs to respond to fluid flow, as 83.8±7.0% of cells responded with mean amplitude of 180.5±20.2nM.

6.3.3 Increasing peak shear stress does not alter calcium signaling

To assess the effect of peak fluid shear stress on fluid flow-induced $Ca^{2+}{}_i$ transients, we exposed hMSCs to a constant flow rate while raising the peak shear stress by increasing the viscosity of the flow media. In control samples, a flow rate of 4.5ml/min inducing a peak shear stress of 5dynes/cm^2 triggered an increase in $[Ca^{2+}]_i$ in 42.8±6.6% of cells with a mean amplitude of 70.4±7.9nM (Figure 6-3A,B). Increasing the viscosity to induce peak shear stresses of 7.8, 12.6, and 20dynes/cm^2 while maintaining a flow rate of 4.5ml/min did not significantly alter the percentage of cells responding (53.1±9.6%, 48.6±6.6%, and 37.6±4.1%, respectively) or the amplitude of the response (67.4±7.9nM, 54.2±5.7nM, and 68.8±11.0nM, respectively). These data suggest that peak fluid shear stress is not a major contributor to the responsiveness of hMSCs to oscillatory fluid flow.

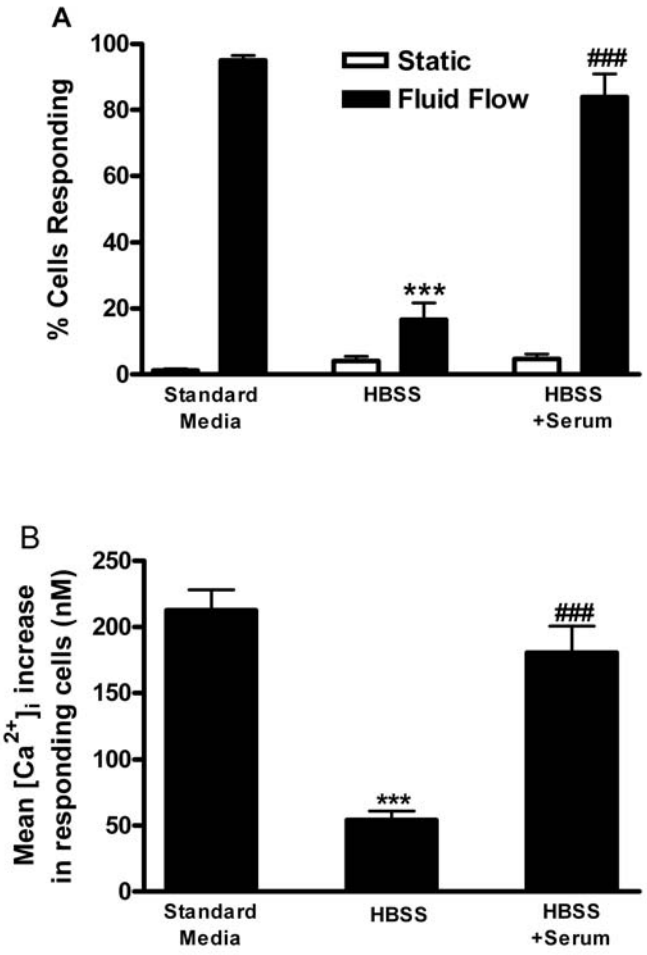

Figure 6-2. Effect of removing chemotransport on calcium signaling. hMSCs were exposed to fluid flow inducing a peak shear stress of 20dyn/cm^2 in standard flow media containing 0.5% FBS, HBSS (devoid of nutrients), or HBSS containing 0.5% FBS, and the percentage of cells responding (A), and the mean increase in $[Ca^{2+}]_i$ in cells responding to fluid flow (B) were quantified. *** $p<0.001$ compared to standard media, and ### $p<0.001$to HBSS.

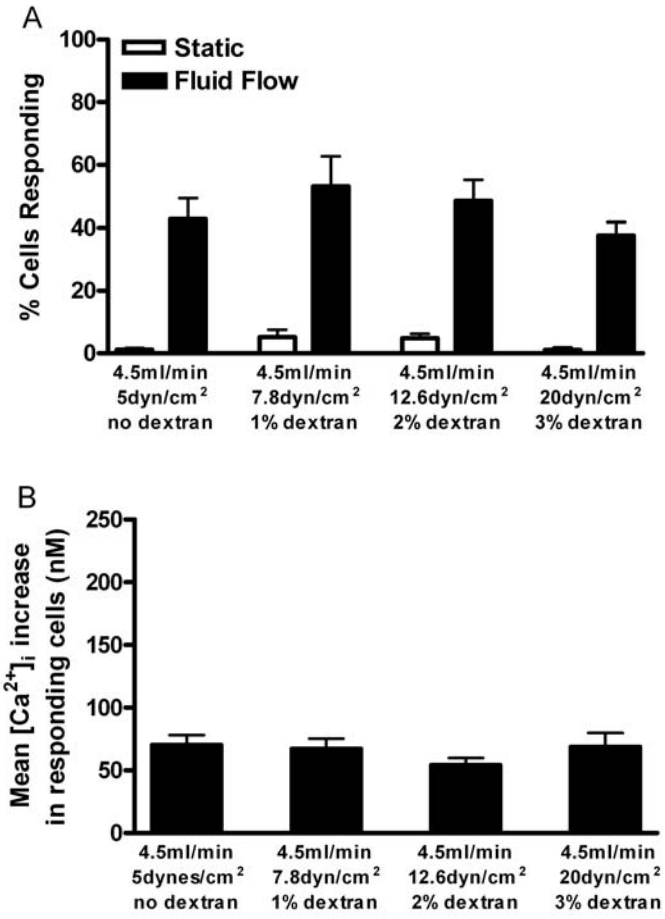

Figure 6-3. Effect of peak shear stress on calcium signaling. Chemotransport was held constant (flow rate: 4.5ml/min), while peak shear stress was increased by adding neutral dextran to flow media, and the percentage of cells responding (A), and the mean increase in $[Ca^{2+}]_i$ in cells responding to fluid flow (B) were examined. A significant difference was not observed in either the percentage of cells responding or the mean increase in $[Ca^{2+}]_i$ in cells exposed to 5, 7.8, 12.6 and 20dyn/cm^2 of fluid shear stress.

6.3.4 Proliferation is decreased with changes in chemotransport.

To examine the effects of chemotransport and fluid shear stress on a long term response to oscillatory fluid flow, we assessed the contribution of these stimuli to cellular proliferation. A flow rate of 56ml/min inducing a peak shear stress of 20dynes/cm^2 significantly increased hMSC proliferation to 234.6±16.3% of static controls (Figure 6-4A). Lowering the flow rate to 37ml/min or 23ml/min while maintaining a peak shear stress of 20dynes/cm^2, abolished the increase in cellular proliferation in response to fluid flow (81.5±14.5% and 94.8±12.2% of no dextran static control). The addition of 1% and 2% dextran to the flow media had no effect on static proliferation levels (93.4±9.2% and 94.0±9.6%, respectively vs 100.0±5.2% for no dextran). These data suggest that chemotransport is also important for the proliferation response of hMSC to fluid flow. Increasing peak fluid shear stress had no detectable effect on hMSC proliferation (Figure 6-4B). A flow rate of 14ml/min inducing a peak shear stress of 5 (no dextran), 7.8 (1% dextran), 12.6 (2% dextran), or 20dynes/cm^2 (3% dextran) did not increase hMSC proliferation (128.9±12.8%, 89.60±15.1%, 105.2±15.0%, 113.0±29.4%, respectively) relative to static controls. Treatment with 20% fetal bovine serum was used as a positive control in these experiments and increased hMSC proliferation to 534.5±28.8% of static controls.

6.3.5 ATP release is altered when chemotransport is decreased

Since we have found that the release of ATP and subsequent activation of purinergic receptors mediates the effect of fluid flow on hMSC calcium responses and cellular proliferation (536), we also examined the contributions of chemotransport and

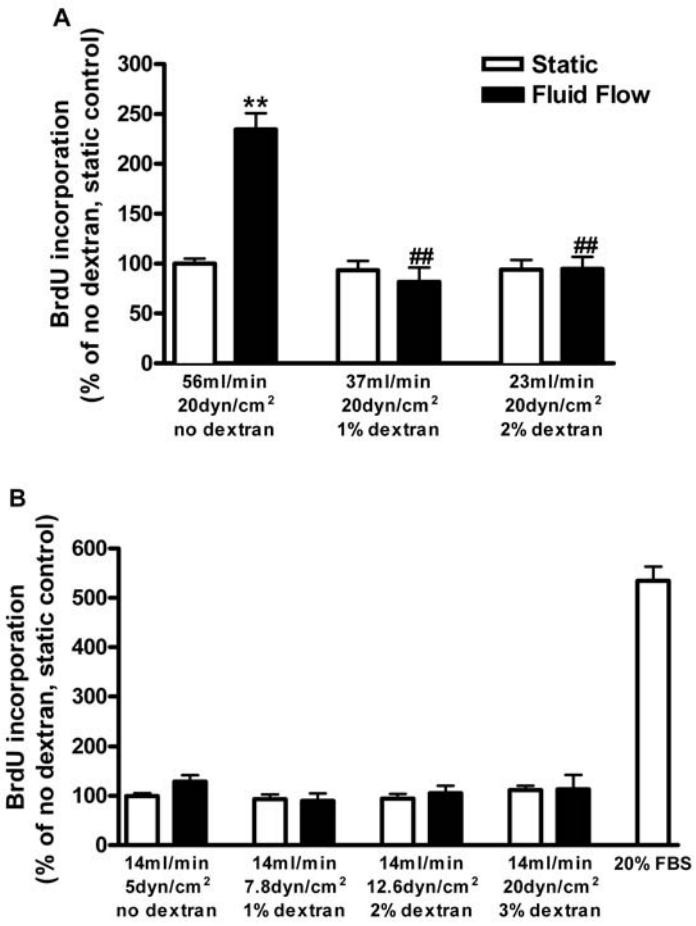

Figure 6-4. Effect of altering chemotransport or peak shear stress on proliferation. hMSCs were exposed to fluid flow for 1hour with peak shear stress (A, 20dyn/cm^2) or chemotransport (B, flow rate: 14ml/min) held constant, or left unflowed as static controls and then returned to culture for 20h in standard flow media. Proliferating cells were labeled with 10μM 5-bromo-2-deoxyuridine (BrdU) and quantified by fluorescent-activated cell sorting. *** $p<0.001$compared to no dextran, static control, and ## $p<0.01$ to 56ml/min, 20dyn/cm^2, no dextran flow.

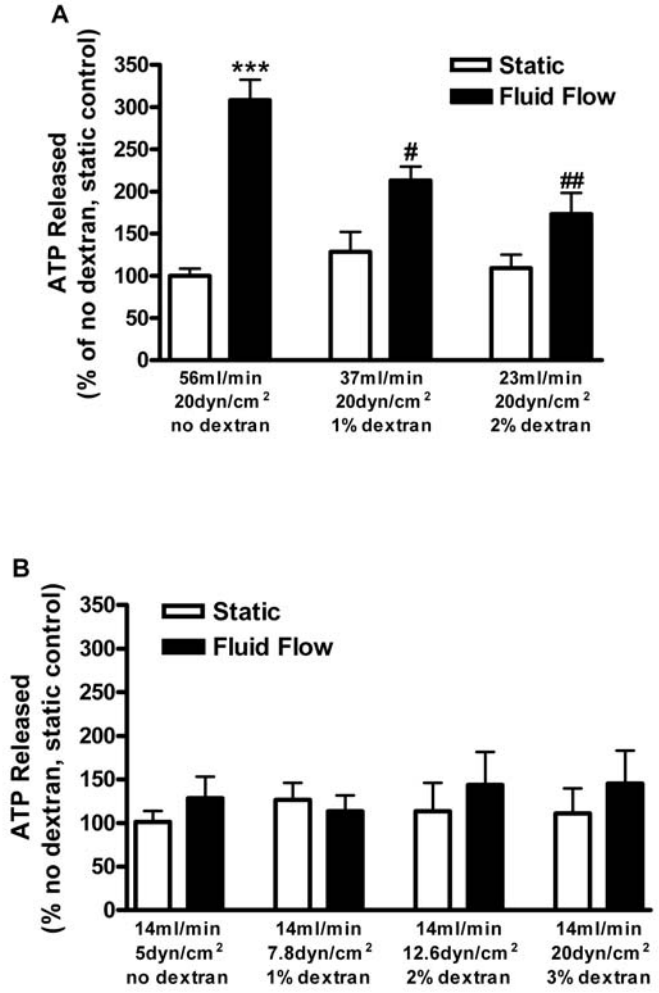

Figure 6-5. Effect of altering chemotransport or peak shear stress on ATP release. hMSCs were exposed to fluid flow for 5min with peak shear stress (A, 20dyn/cm^2) or chemotransport (B, flow rate: 14ml/min) held constant, or left unflowed as static controls. Condition media samples were collected and analyzed for ATP concentration using a luciferin:luciferase based reaction. *** $p<0.001$ compared to no dextran, static control, # $p<0.05$ to 56ml/min, 20dyn/cm^2, no dextran flow, and ## $p<0.01$ to 56ml/min, 20dyn/cm^2, no dextran flow.

fluid shear stress on ATP release. A flow rate of 56ml/min inducing a peak shear stress of 20dynes/cm^2 increased the release of ATP from hMSCs to 308±23.8% of static controls (Figure 6-5A). Lowering the flow rate from 56ml/min to 37ml/min or 23ml/min significantly decreased ATP release from hMSCs to 212.9±16.7% and 173.1±25.0% of no dextran, static controls. The addition of neutral dextran to the flow media at concentrations of 1% and 2% did not affect the release of ATP in static cultures (128.5±23.8% and 109.1±15.9%, respectively, vs 100±8.5%). Similar to intracellular calcium signaling and cellular proliferation, altering peak shear stress had no effect on ATP release from hMSCs (Figure 5B). A flow rate of 14ml/min inducing at peak shear stress of 5 (no dextran), 7.8 (1% dextran), 12.6 (2% dextran), or 20dynes/cm^2 (3% dextran) did not increase ATP release (128.6±24.6%, 113.5± 18.1%, 143.6±37.4% and 145.0±38.1%, respectively).

6.3.6 Osteoblastic differentiation does not alter responsiveness to chemotransport and fluid shear stress

As previous studies examining the contributions of chemotransport and fluid shear stress utilized models of more mature osteoblastic cells (6,170,214,215), we examined the effect of differentiating hMSCs along the osteoblastic lineage on responses to chemotransport and peak fluid shear stress. Treating hMSCs for 12days with 10nM dexamethasone, 10mM β-glycerol phosphate, and 50μg/ml ascorbic acid phosphate significantly increased the activity of alkaline phosphatase, a phenotypic marker of osteoblastic cells, compared to undifferentiated controls (84.6±4.4 vs 335.7±12.2 nM p-nitrophenol produced/min/mg of protein, Figure 6-6A). However, the osteoblastic differentiation of hMSCs failed to alter sensitivity to changes in chemotransport or peak shear stress in response to oscillatory fluid flow compared to undifferentiated cells

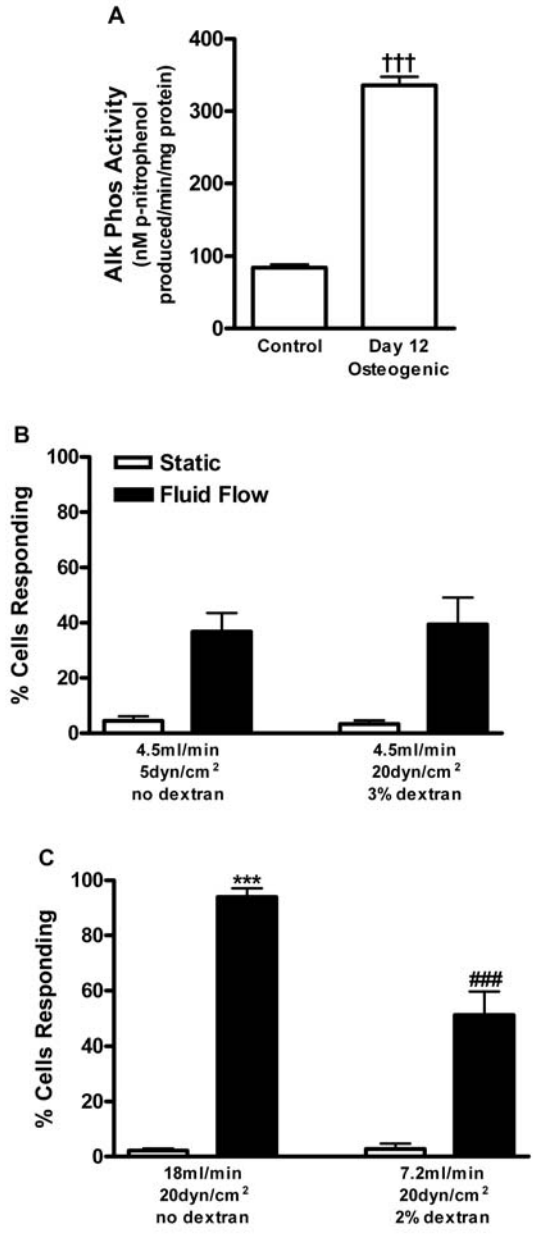

Figure 6-6. Effect of osteoblastic differentiation on responses to altering peak shear stress and chemotransport. hMSCs were differentiated along the osteoblastic lineage for 12days and the activity of alkaline phosphatase compared to undifferentiated controls (A). Osteoblastic cells were then exposed to oscillatory fluid flow with chemotransport held constant (flow rate: 4.5ml/min) and peak shear stress altered (B) or peak shear stress held constant (20dynes/cm^2) and chemotransport altered (C) and the percentage of cells responding with a change in intracellular calcium concentration assessed. ††† $p<0.001$ compared to undifferentiated control, *** $p<0.001$ to no dextran, static control, and ### $p<0.001$ to from 18ml/min, 20dynes/cm^2.

(Figure 6B,C). A flow rate of 4.5ml/min, inducing a peak shear stress of 5dynes/cm^2 stimulated an increase in $[Ca^{2+}]_i$ in 36.8±6.7% of osteogenic hMSCs, while 39.3±9.8% of cells responded when the peak shear stress was increased to 20dynes/cm^2. Further, decreasing the flow rate from 18ml/min to 7.2ml/min while maintaining a peak shear stress of 20dynes/cm^2 decreased the number of cells responding to fluid flow with an increase in $[Ca^{2+}]_i$ from 94.0±3.1% to 51.09±8.6%.

6.3.7 hMSCs are more responsive to steady fluid flow

Finally, in light of these findings, we hypothesized that hMSCs would be more responsive to steady fluid flow compared to oscillatory fluid flow due in part to the increased chemotransport inherent in this flow regime. hMSCs were exposed to an oscillatory or steady fluid flow regime with a flow rate of 4.5ml/min and peak shear stress of 5dynes/cm^2 and changes in $[Ca^{2+}]_i$ were examined. As indicated above, 42.8±6.6% of cells responded to oscillatory fluid flow with a mean increase in $[Ca^{2+}]_i$ of 70.4±7.9nM (Figure 3A,B, Figure 7A,B). In response to steady fluid flow, 94.5±4.1% of cells responded with a mean increase in $[Ca^{2+}]_i$ of 160.2±23.5nM, indicating that hMSCs are significantly more responsive to steady flow than oscillatory flow. To determine whether this increased responsiveness was related to increased levels of chemotransport, we repeated these experiments in HBSS. Under these conditions, 48.6±12.1% of cells responded to steady flow with a mean increase in $[Ca^{2+}]_i$ of 39.9±6.0nM.

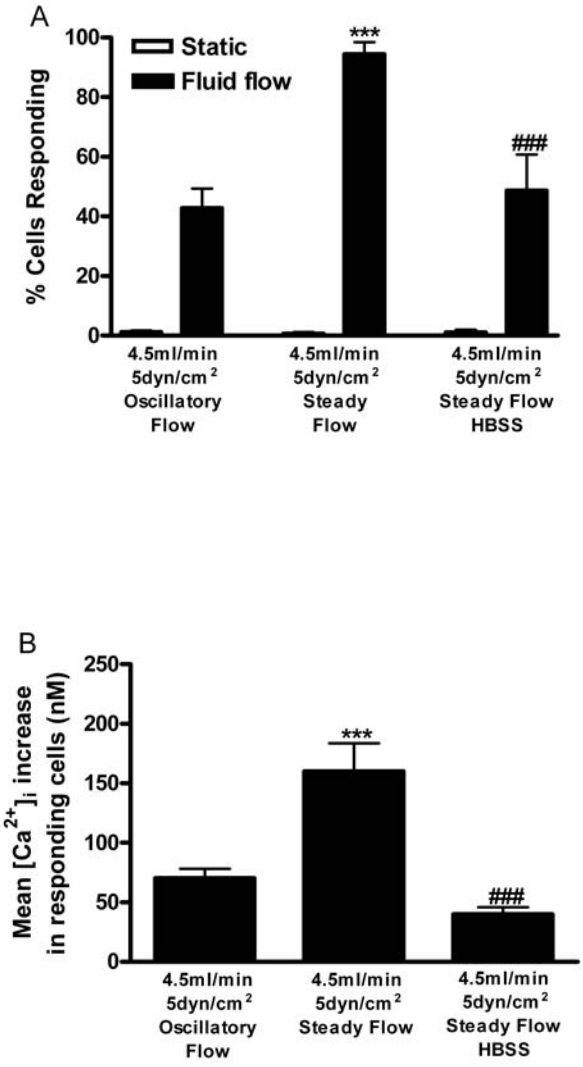

Figure 6-7. Comparison of steady and oscillatory fluid flow effects on calcium signaling. hMSCs were exposed to steady or oscillatory fluid flow with a flow rate of 4.5ml/min inducing a peak shear stress of 5dyn/cm^2 in standard flow media or HBSS and the percentage of cells responding (A), and the mean increase in $[Ca^{2+}]_i$ in cells responding to fluid flow (B) were quantified. *** $p<0.001$ compared to oscillatory fluid flow, and ### $p<0.001$ to steady fluid flow in standard media.

6.4 Discussion

Interstitial fluid flow resulting from the deformation of skeletal tissue in response to mechanical load induces a number of biophysical stimuli that bone cells may perceive, including streaming potentials, fluid shear stress, and chemotransport. Previous reports on the contribution of peak fluid shear and chemotransport to bone cell mechanotransduction have been contradictory, as an equal number of studies support the importance of each stimulus (6,170,214,215). We would suggest that the discrepancy in these results is likely due to the flow regimes applied. In steady or pulsatile flow regimes, which support the hypothesis that bone cells respond to changes in fluid shear stress, chemotransport is constant. Fresh media is continuously perfused over cell monolayers removing waste products and refreshing nutrient levels. In oscillatory flow regimes, which more closely mimic the movement of interstitial fluid as a result of dynamic loading (4,517), the levels of chemotransport are dependent upon the flow rate utilized. Nutrient replacement and removal of cellular waste depend upon the mixing of flow media with fresh media outside of the chamber, with a higher flow rate leading to greater mixing.

In Chapter 4 and 5, we found that human mesenchymal stem cells respond to fluid flow in a flow-rate dependent manner, but we did not distinguish between the effects of peak fluid shear stress and chemotransport (513,536). In this study, we found that chemotransport, but not fluid shear stress, is a determining factor in the responses of hMSCs to fluid flow. Increases in cellular proliferation, intracellular calcium concentration, and ATP release were all diminished when the levels of chemotransport were attenuated by lowering the flow rate utilized in these experiments. Conversely,

incrementally increasing fluid shear stress from 5 to 20dynes/cm^2 was not sufficient to alter these same responses. Given the anatomical location of hMSCs within the bone marrow these results are not unexpected. While it is likely that hMSCs are exposed to fluid flow *in vivo*, it is unclear whether hMSCs within the marrow cavity would experience significant levels of fluid shear stress. Current estimates of the physiological levels of fluid shear are based upon the geometry of the lacunar-canalicular system (5,208,538,539) or haversian systems (209), but hMSCs are not confined to such spaces. We would hypothesize and our data suggests that hMSCs are more likely to experience load-induced fluid flow in the form of chemotransport. Further support for this hypothesis is drawn from a report by Sikavitsas *et al*. (515) that found no effect of fluid shear stress on rat bone marrow stromal cell proliferation when a steady fluid flow regime was utilized.

Two possible mechanisms by which chemotransport may contribute to hMSC sensitivity to fluid flow are through the removal of cellular waste products that might act as inhibitory molecules or through the replacement of nutrient levels. The inability of hMSCs to respond to fluid flow with an increase in intracellular calcium concentration in Hank's balanced salt solution, which has also been observed by Haut Donahue *et al*. (6) and Allen *et al*. (214) in osteoblastic cell models, suggests the latter mechanism is more likely. HBSS lacks the amino acids or glucose present in standard flow media, but cellular waste products would still be removed with fluid flow exposure. Interestingly, adding serum to HBSS rescued responsiveness to fluid flow. The interpretation of these results in previous studies (6,11,214), was that a biochemical factor in serum, likely ATP as asserted by You *et al*. (11), is necessary for bone cell mechanotransduction. More

recent evidence suggests that bone cells release ATP in response to fluid flow (7,10,239,536). An intriguing hypothesis is that the transport of nutrients contributes to the release of ATP in response to fluid flow. The cellular uptake of amino acids induces hypotonic swelling (540,541) which can trigger the release of ATP (542-544). Indeed, osmotic loading is a well established model of chondrocyte mechanotransduction (545-547) and Romanello *et al*. (240,241) have already reported that cell swelling induces the release of ATP from bone cells. Whether exposing hMSCs to fluid flow induces cell swelling and this contributes to ATP release will require further examination.

As previous studies suggested that osteoblastic cell models are responsive to changes in peak shear stress (170,215), we differentiated hMSCs along the osteoblastic lineage to determine whether sensitivity to chemotransport and an insensitivity to peak shear stress is unique to hMSCs. Surprisingly, we found that even after 12 days of osteoblastic differentiation cells exhibit similar mechanosensitivity and were more responsive to changes in chemotransport than to peak fluid shear stress. One possible explanation of these data is that that bone cells acquire sensitivity to changes in fluid shear stress only after terminal differentiation. However, the shear-dependent effects described in osteoblastic cells exposed to steady and pulsatile flow suggest otherwise (170,215). A second possibility is that these results highlight a difference between oscillatory fluid flow regimes, where chemotransport depends upon the flow rate, and steady fluid flow regimes, where chemotransport is constant and cells perceive changes in fluid shear stress.

Finally, we found that hMSCs were much more responsive to a steady fluid flow regime compared to an oscillatory fluid flow regime. Whereas approximately 45% of

hMSCs responded to oscillatory fluid flow with a flow rate of 4.5ml/min with an increase in intracellular calcium concentration, nearly 95% of hMSCs responded to steady fluid flow with the same flow rate. These results are similar to a previous report by Jacobs *et al*. (517) that found immortalized human fetal osteoblasts (hFOB 1.19) to be more responsive to steady fluid flow compared to oscillatory fluid flow. Importantly, we found that this increase in responsiveness was due to enhanced chemotransport. The percentage of hMSCs responding to steady fluid flow in HBSS was dramatically decreased compared to standard media. Given the intense interest in utilizing hMSCs in tissue engineering (322,323), and the use of mechanical signals in directing cell proliferation and differentiation, these data would suggest that steady fluid flow may be a more effective treatment than the more physiologically relevant oscillatory fluid flow in optimizing these protocols.

In summary, this study outlines the effect of chemotransport and peak fluid shear stress on human mesenchymal stem cell proliferation in response to fluid flow. We demonstrate that the flow rate-dependent effects of oscillatory fluid flow on hMSC proliferation (513) are mediated by alterations in chemotransport, but not peak fluid shear stress. Further, this effect likely results from similar dynamics in the release of ATP and intracellular calcium signaling. These data imply that removal of cellular waste and sufficient nutrient supply are important mediators of hMSC mechanotransduction and will be important factors in tissue engineering protocols designed to direct the proliferation and differentiation of this cell population.

CHAPTER 7

CONCLUSIONS AND REMAINING QUESTIONS

7.1 Overview

Bone marrow-derived mesenchymal stem cells have the potential to support the differentiation of many cell lineages including those of the osteoblast, adipocyte, and chondrocyte (282-285). As such, there is a great interest in understanding how this cell population contributes to the pathophysiology of bone diseases and identifying factors that stimulate proliferation and differentiation for use in therapeutic applications. Already, a number of transcription factors, growth factors, and other extracellular cues that direct the lineage determination of mesenchymal stem cells have been identified. Despite the well established effects of mechanical signals on bone, the effects of these signals on mesenchymal stem cells are relatively unexplored. To examine the effects of biophysical signals on the hMSCs, the specific aims of this thesis were three-fold: 1) to examine the effect of oscillatory fluid flow on hMSC proliferation and identify the signaling cascades necessary for this response; 2) to examine the signal(s) that initiates the activation of these signaling events; and, 3) to identify the biophysical signal by which hMSCs perceive changes in their mechanical environment.

7.2 Conclusions

7.2.1 Oscillatory fluid flow induces hMSC proliferation

Stem cells represent only a small fraction of the cells within a tissue. Estimates of the percentage of MSCs among bone marrow cells are on the order of 0.001% to 0.01% (284). Thus, to exert an effect on tissue homeostasis or repair, stem cells must first respond to anabolic signals by proliferating. This ensures the maintenance of stem cell numbers (self-renewal) while increasing the number of effector cells. Aubin (548) suggests MSCs divide eight to ten times before obtaining a differentiated phenotype. A complete understanding of the factors that regulate MSC proliferation could have

important implications in the treatment of metabolic bone diseases, like osteoporosis, as well as the amplification of stem cell numbers for use in tissue engineering.

Anecdotal evidence suggests that MSC proliferation and subsequent differentitation is regulated by mechanical signals. Following periods of skeletal unloading, MSCs isolated from experimental models form fewer and smaller osteogenic colonies implying a deficit in proliferation potential (445-447,549). Our studies provide direct evidence that exposing hMSCs to a physiologically-relevant mechanical signal regulates proliferation. Exposing hMSCs to oscillatory fluid flow induced a flow rate-dependent increase in proliferation. Flow rates sufficient to induce shear stresses of 5 or 10dynes/cm^2 had no effect on hMSC proliferation, but a flow rate inducing a shear stress of 20dynes/cm^2 significantly increased hMSC proliferation (Figure 4-5). These results confirm the findings of Li *et al.* (452), and suggest that a critical threshold must be meet for fluid flow to stimulate proliferation.

We found that increases in hMSC proliferation in response to fluid flow are dependent upon the activation of MAP kinase signaling and calcium signaling pathways. Fluid flow exposure induced the activating, phosphorylation of ERK1/2 (Figure 4-3) and pharmacological inhibition of ERK1/2 activation inhibited fluid flow-induced proliferation. Similarly, fluid flow stimulated a transient increase in intracellular calcium concentration (Figure 4-1) and the activation of calcineurin (Figure 4-4) and pharmacological inhibition of this protein inhibited fluid flow-induced proliferation. Interestingly, while intracellular calcium signaling was dependent upon the flow rate, ERK1/2 phosphorylation was increased at all flow rates utilized. While Kapur *et al.* (265) reported that the activation of both ERK1 and ERK2 are necessary for fluid flow to

induce osteoblast proliferation, our data suggest that calcium signaling is the primary signal determining the effect of fluid flow on hMSC proliferation.

7.2.2 ATP activates calcium signaling pathways to induce hMSC proliferation

A growing body of evidence suggests that purinergic signaling is an important mediator of bone cell mechanotransduction. The expression of purinergic receptors has been reported in both osteoblasts and osteocytes (242,511) and alterations in receptor expression are associated with alterations in mechanosensitivity. Mice lacking the $P2X_7$ receptor exhibit decreased sensitivity to mechanical signals leading to a decrease in periosteal bone formation rate and an increase in trabecular bone resorption (10,512). Similarly, You *et al*. (11) reported that the activation of the $P2Y_2$ receptor mediates the effect of fluid flow on intracellular calcium signaling. Both osteoblastic and osteocytic cell models have also been shown to release ATP in response to fluid flow exposure (7,239).

In these studies, we hypothesized that purinergic signaling also mediates the effects of fluid flow on hMSC proliferation. Consistent with this hypothesis, hMSCs expressed several purinergic receptors, most notably the $P2Y_2$ and $P2X_7$ receptors already implicated in mechanotransduction (Figure 5-1) (10,11). Additionally, hMSCs released ATP in response to fluid flow exposure (Figure 5-2) and treatment with exogenous ATP increased hMSC proliferation (Figure 5-3). Interestingly, we found that the release of ATP is flow rate-dependent and likely explains the flow rate-dependent effect of fluid flow on proliferation and calcium signaling (Figure 5-2). Flow rates inducing shear stresses of 5 and 10dynes/cm^2 that did not induce hMSC proliferation or robust increases in intracellular calcium concentration, also failed to induce ATP release. However, a

flow rate inducing a shear stress of 20dynes/cm^2 induced both ATP release and hMSC proliferation. Enzymatically degrading this molecule abolished the effect of fluid flow on hMSC proliferation as well as the activation of the calcium signaling cascade leading to this response (Figure 5-4).

To the best of our knowledge, these are the first studies to identify a potential mechanism for the activation of calcineurin/NFAT signaling in osteoblasts. Many studies have implicated calcineurin and NFAT in the differentiation of osteoclastic cells (550-553), but these signaling pathways have only recently been studied in osteoblastic cells (260,261,554). Further, these studies did not examine the mechanism by which calcineurin/NFAT signaling might be activated in cells of the osteoblast lineage. Our studies demonstrate that purinergic signaling represents one mechanism by which this signaling pathway may be activated. We found that treating hMSCs with exogenous ATP activated calcineurin and induced the nuclear localization of NFAT, while degrading ATP abolished these same responses after fluid flow exposure (Figure 5-8).

7.2.3 hMSCs perceive oscillatory fluid flow in the form of chemotransport

An almost dogmatic assumption in the study of bone cell mechanotransduction is that cells perceive interstitial fluid flow in the form of fluid shear stress. Based upon measurements of load-induced streaming potentials, Weinbaum *et al*. (5) calculated fluid shear stresses to be in the range of 8 to 30dynes/cm^2 for osteocytic cells. Since this time, most studies, including the work presented here, have utilized fluid flow regimes sufficient to induce fluid shear stresses within this range regardless of the cell model. It is possible that bone cells perceive interstitial fluid flow via other mechanisms like streaming potentials and chemotransport. Indeed, studies by Allen *et al*. (214) and Haut

Donahue *et al*. (6) suggest that chemotransport contributes to the effects of fluid flow on intracellular calcium signaling and prostaglandin production.

The data presented in Chapter 6 suggest that hMSCs perceive interstitial fluid flow via chemotransport effects. Incrementally increasing the peak fluid shear stresses from 5 to 20dynes/cm^2 while maintaining a constant flow rate had no effect on the parameters we examined, including cellular proliferation (Figure 6-4), intracellular calcium signaling (Figure 6-3), and release of ATP (Figure 6-5). Conversely, decreasing the flow rate, while maintaining a peak fluid shear stress of 20dynes/cm^2, decreased the ability of hMSCs to respond to fluid flow. Decreasing the flow rate abolished the effect of fluid flow on cellular proliferation (Figure 6-4) while decreasing the number of cells responding the fluid flow with an increase in intracellular calcium (Figure 6-1) and the amount of ATP released (Figure 6-5). Further, when we completely removed chemotransport by exposing hMSCs to fluid flow in Hank's balanced salt solution, lacking amino acids or serum, the ability of hMSCs to respond with an increase in intracellular calcium concentration was nearly abolished (Figure 6-2).

While these data represent a divergence from dogma, they are not entirely unexpected. MSC presumably do not occupy the same confined geometry in which osteocytes reside and as such, the levels of fluid shear stress calculated by Weinbaum *et al*. (5) are probably not applicable. Some migratory progenitor cells may experience significant levels of fluid shear stress in haversian systems (209) or in a remodeling osteon (464,465), but the vast majority of MSCS are likely to perceive interstitial fluid flow in another form, possibly chemotransport. It is intriguing that MSCs did not acquire sensitivity to fluid shear after osteogenic differentiation (Figure 6-6), as both Haut

Donahue *et al*. (6) and Allen *et al*. (214) found that some responses to fluid flow could be modulated by increases in fluid shear stress in osteoblasts. Whether this discrepancy results from differences in the cell model utilized (i.e. mouse vs. human, or primary vs. transformed cell lines) remains be determined, but suggests that further investigation of the effects of chemotransport in bone cell mechanotransduction is necessary.

7.3 Proposed model for fluid flow-induced hMSC proliferation

Figure 7-1 illustrates our model for the induction of hMSC proliferation by oscillatory fluid flow. The initiation of fluid flow replaces nutrient levels in the cells viscinity (chemotransport) and the uptake of molecules such as amino acids and glucose stimulates cell swelling (Figure 7-1A). An increase in cell volume triggers the activation of a calcium channel that produces a localized increase in intracellular calcium sufficient to induce the vesicular release of ATP (Figure 7-1B). Extracellular ATP activates purinergic receptors on the cell surface inducing an increase in intracellular calcium concentration. Since our data suggest that the release of calcium from IP_3-sensitive stores contributes to this response, we hypothesize that this is a P2Y receptor (Figure 7-1C). Increased intracellular calcium levels activate calcineurin which dephosphorylates NFAT allowing it to translocate to the nucleus and induce the expression of cell cycle-related genes (Figure 7-1D).

As we have not identified the mechanism by which oscillatory fluid flow stimulates ERK1/2 phosphorylation, MAP kinase signaling was purposely excluded from this model. Potential mechanisms for the activation of MAP kinase signaling are discussed below.

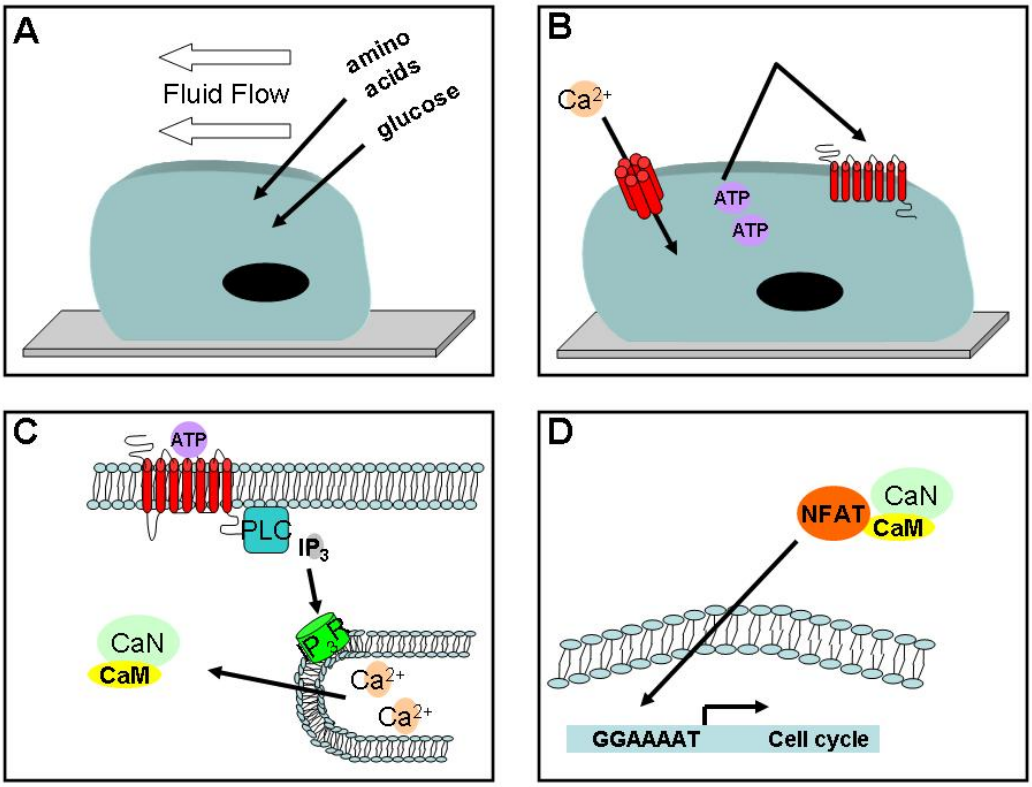

Figure 7-1 Model for fluid flow-induced hMSC proliferation

7.4 Implications

The motivation for these studies partially stems from the perceived alteration in MSC proliferation and differentiation following skeletal unloading and in many metabolic bone diseases. As discussed in Chapter 3, skeletal disuse and age-related bone loss are accompanied by an increase in bone marrow adiposity (303,305,306). The current hypothesis suggests that this observation represents an increase in the adipogenic differentiation of MSCs at the expense of osteoblastic differentiation (307). While we have focused on proliferation, our results have implications for the differentiation of MSCs as many of the the signaling cascades that are activated by fluid flow also regulate differentiation. Regarding adipocyte differentiation, the activation of calcineurin and nuclear translocation of NFATc1 inhibits the early stages of differentiation by inhibiting the expression of adipogenic transcription factors (534,555). Similarly, the activation of MAP kinase signaling inhibits the transcriptional activity of PPARγ and also inhibits adipocyte differentiation (479,556-558). Conversely, these same pathways enhance osteoblastic differentiation. NFATc1 acts synergistically with Osterix to enhance type I collagen expression (554), while the activation of MAP kinase signaling increases CBFA1 transcriptional activity (367) and following fluid flow exposure enhances the expression of bone matrix proteins (210,211). Thus, fluid flow may negatively regulate adipogenesis while enhancing osteogenesis.

A second motivation stems from the interest in utilizing hMSCs in tissue engineering. While multipotent cells have been isolated from numerous tissues (271-274), the *in vitro* cultivation of these cells still represents a major hurdle as prolonged culture is associated with a decline in proliferation and differentiation capacity (338,339). Additionally,

current cultivation techniques risk the development of an immune response following transplantation (340,341). These studies suggest that fluid flow or ATP treatment can be utilized to stimulate hMSC proliferation *in vitro*, while reports by Li *et al*. (452) and Kreke *et al*. (453,454), suggest fluid flow can be used to augment the osteoblastic differentiation of hMSCs. Indeed, the application of fluid flow to scaffolds containing MSCs enhances extracellular matrix production and calcium deposition (514). Our data suggest that while oscillatory fluid flow represents the more physiologically relevant flow regime (517), steady flow may be better suited for these practices. Like osteoblastic cells (517), hMSCs were much more responsive to steady fluid flow than an oscillatory fluid flow regime likely due to the increase in chemotransport (Figure 6-7). Whether fluid flow exposure helps to maintain the differentiation potential of hMSC after *in vitro* propagation remains to be seen.

7.5 Remaining Questions

7.5.1 What P2 receptor mediates the effect of fluid flow on proliferation?

In chapter 5, we found that the release of ATP and subsequent activation of purinergic receptors is necessary for fluid flow to induce hMSC proliferation, but we did not identify the receptor(s) responsible for this effect. Figure 5-1 reveals that hMSCs express several purinergic receptors including $P2Y_2$, $P2Y_6$, $P2Y_{11}$, and $P2X_7$. While the $P2X_7$ receptor has already been implicated in bone cell mechanotransduction (10), our data suggest that a P2Y receptor is involved in the effect of fluid flow on hMSC proliferation. The activation of phospholipase C and release of calcium from IP_3 sensitive stores contributed to the increase in intracellular calcium concentration necessary for fluid flow to stimulate proliferation (Figure 4-2) and this is more likely to be related to the activation of a metabotropic P2Y receptor than an ionotropic P2X

receptor (559). The effects of exogenous nucleotides on hMSC proliferation in static cultures further limit the number of candidate receptors. The observation that ATP but not other nucleotides (most notably UTP, Figure 5-3) enhanced hMSC proliferation suggests flow-induced proliferation may be mediated by the $P2Y_{11}$ receptor. If this is indeed the case then it would be interesting to examine whether the activation of adenylate cyclase and the production of cAMP is also necessary for fluid flow to stimulate hMSC proliferation, as the activation of $P2Y_{11}$ stimulates both adenylate cyclase activity and phospholipase C activity (560,561). However, we found that both ATP and UTP stimulate an increase in intracellular calcium concentration in hMSCs (Figure 5-6) suggesting that $P2Y_2$ receptors could also be activated by fluid flow. It is possible that the activation of both the $P2Y_2$ and $P2Y_{11}$ receptors are necessary for fluid flow to stimulate hMSC proliferation.

7.5.2 What role does NFAT play in inducing proliferation?

While we have found that the activation of calcineurin is necessary for fluid flow to induce hMSC proliferation (Figure 4-5), it remains unclear what role NFAT plays in this response. Two possible mechanisms exist. First, NFAT may directly regulate the expression of cell cycle-related genes. In support of this hypothesis, binding sites for NFAT have been identified in the promoter regions of cyclin-dependent kinase 4 (533,562) and cyclin D1 (562). Our own preliminary data suggests that expression levels of these proteins are increased by fluid flow. Second, NFAT may regulate the expression of an intermediate factor that acts an autocrine/paracrine regulator of cell proliferation. In 3T3-L1 pre-adipocytes, NFATc1 regulates cell cycle progression by enhancing the expression of a secreted, heat-liable factor (534). Similarly, Winslow *et al*. (260) found

that expression of a constitutively active NFATc1 induces a high bone mass phenotype in mice as a result of enhanced osteoblastic proliferation and this effect may be mediated by the enhanced expression of Wnt signaling components. Chromatin immunoprecipitation experiments could help determine what genes NFAT regulates after fluid flow exposure.

7.5.3 How does chemotransport activate hMSCs?

In Chapter 6, we found that chemotransport, the removal of cellular metabolites and replacement of nutrient levels, is the primary signal that hMSCs perceive in response to fluid flow. While others have also reported the involvement of chemotransport in bone cell mechanotransduction (6,214), a clear mechanism for the activation of cell signaling events by chemotransport has not been established. Our hypothesis is that chemotransport regulates hMSC proliferation by affecting cell volume. As depicted in our model (Figure 7-1), fluid flow replaces and stimulates the uptake of amino acids and/or glucose leading to an increase in cell volume. Changes in cell volume have already been shown in several cell models to induce the responses necessary for fluid flow to induce hMSC proliferation. Romanello *et al* (240,241) reported that exposing an osteoblastic cell model to hypotonic solutions that induce cell swelling stimulates ATP release and an increase in intracellular calcium concentration. Similarly, osmotic loading is a well established model of chondrocyte mechanotransduction (545-547). In hepatocytes, the uptake of amino acids stimulates an increase in cell volume (540,541) that stimulates the release of ATP (544,563). We have not determined whether fluid flow exposure increases hMSC cell volume, but preliminary experiments suggest that the addition of amino acids to Hank's balanced salt solution rescues the ability of hMSCs to respond to fluid flow with an increase in intracellular calcium concentration.

7.5.4 What signal induces ERK1/2 phosphorylation?

We found that oscillatory fluid flow stimulates the phosphorylation of the MAP kinases ERK1/2 (Figure 4-3), but we did not identify the signal that induces this response. Our initial hypothesis was that purinergic signaling mediates the activation of ERK1/2 like intracellular calcium signaling, but this was not the case. While exogenous ATP stimulated ERK1/2 phosphorylation, treating hMSCs with apyrase did not inhibit fluid flow-induced phosphorylation (Figure 5-9). A number of mechanisms could be responsible for the activation of MAP kinase signaling in response to fluid flow. Weyts *et al* (228) suggested that ERK1/2 activation in response to fluid flow may proceed via the activation of integrin signaling. Indeed, when osteoblastic cells were are transfected with mutated forms of focal adhesion kinase or proline-rich tyrosine kinase 2, components of the integrin signaling pathway, ERK1/2 phosphorylation in response to mechanical stimulation was abolished (535). Integrin signaling could contribute to the effect of fluid flow on ERK1/2 phosphorylation in hMSCs, but our data suggests that PKC would also be involved. Treating cells with calphostin C abolished the phosphorylation of ERK1/2 in response to fluid flow (Figure 4-3). In endothelial cells, fluid flow also stimulates ERK1/2 phosphorylation via the activation of PKC (564), but it remains unclear what signal is upstream of PKC. We found that the release of calcium from intracellular stores does not stimulate ERK1/2 phosphorylation, but it is possibility that fluid flow stimulates the activation of a calcium-conducting channel that activates PKC and subsequently ERK1/2. Jessop *et al*. (493) found that antagonizing the L-type calcium channel abolished ERK1/2 phosphorylation in ROS17/2.8 cells, but treating

hMSCs with verapamil did not produce the same effects. It remains possible that the activation of another calcium channel activates MAP kinase signaling.

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Refereed Publications

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Book Chapters

Riddle RC, Taylor AF, Donahue HJ. Fluid Flow Assays. In: Osteoporosis - Methods in Molecular Medicine (Westendorf, JJ ed) Humana Press (**In Press**).