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| Feature Article |

Molecular Responses in Osteogenic Differentiation of Mesenchymal Stem Cells Induced by Physical Stimulation

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Abstract : Mesenchymal stem cells (MSCs) have been recognized as a great source of stem cells in the field of regenerative medicine and regulation of MSCs such as differentiation into specific cells. Particular interest is the use of physical stimulation for the expression of the osteoblast-specific genes from MSCs for bone tissue regeneration. The mechanical forces on MSCs, such as fluid flow, enhance the mineralized matrix and specific gene expressions. This process called mechanotransduction comprises of the steps of mechanoreception, biochemical coupling, transmission of signal and effector cell response. Physical stimuli effectively regulate extracellular and intracellular signaling pathways to enhance the expression of specific transcription factors, and the release of osteocytes, ultimately expedite the production of active osteoblasts. Thus understating, identification and functional characterization of the mechanotransduction underlying the physical stimulation of MSCs is a critical issue for devising new bone regenerative treatments for bone-related diseases. In this review, we focus on the molecular mechanism responsible for the mechanotransduction of osteogenic differentiation of MSCs induced by physical stimulation.

Key words: *physical stimulation, mechanotransduction, signal transduction, gene expression, osteogenic differentiation*

1. Introduction

Bone formation and resorption are locally regulated by cytokines and growth factors released from the matrix, and systemically regulated by hormones, such as parathyroid hormone and estrogen. Mechanical loading stimulates an anabolic response in osteoblasts by acting in concert with these factors. The mechanism of this response, termed mechanotransduction, comprises the detection of the physical stimulus by the cell (mechanoreception, mechanocoupling), transformation of this stimulus into a biochemical signal (biochemical coupling), and intracellular signal transduction to the nucleus, wherein gene transcription is modified. In the signal transmission process, osteocytes carry out an important function by releasing molecular factors, such as nitric oxide (NO) and prostaglandins (PGs), during the early response to mechanical loading.[1] Mechanical signals are important regulators of skeletal homeostasis, and fluid flow-induced shear stress is a potent mechanical stimulus. It is well known that bone stem cells are exposed to a complex environment that exerts several types of mechanical stress.[2] Bone stem cells are able to detect and respond to shear stress through mechanotransduction, a complex process that converts mechanical forces exerted on a cell into biochemical signals and integrates these into a cellular response. Physical stimulation of cells activates various signal transduction pathways and initiates an anabolic response in osteocytes and osteoblasts leading to changes in gene expression

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and increased cell proliferation and differentiation.[3-5]

The molecular mechanism in osteogenic differentiation of mesenchymal stem cells (MSCs) induced by physical stimulation may influence both mechanoreception (e.g., plasma membrane receptors, ion channels, integrins/focal adhesions and protein kinase signaling) and cellular response (e.g., intracellular calcium (Ca^{2+}), NO, prostacyclin (PGI_2) and cytoskeletal remodeling).[6] Responsiveness of human bone marrow stromal cells to shear stress regulates some steps of many major signaling pathways which can influence the expression and activation of transcriptional factors such as runt-related transcription factors (Runx2) and activator protein 1 (AP-1).[7] These transcription factors can subsequently initiate the transcription of specific genes related to osteogenic differentiation (alkaline phosphatase (ALP), collagen I (Col-I), osteocalcin (OCN), and osteopontin (OPN)). Therefore the purpose of this review is to identify the molecular mechanisms that promote osteogenic differentiation of MSCs.

2. Behavior of MSCs Induced by Physical Stimulation

Mechanical forces on cells such as fluid flow, enhance the mineralized matrix and gene expression of osteoblast-specific genes in MSCs.[8] Holtolf *et al*. (2005) demonstrated that flow perfusion culture can induce osteogenic differentiation of rat MSCs, even in the absence of the osteogenic culture medium supplemented with dexamethasone. They found that dexamethasone and flow perfusion culture had a synergistic effect on osteogenic differentiation.[9] Zhao *et al*. (2005) and Lim *et al*. (2009) also reported that the constructs grown in the perfusion system have uniform cell density and maintain their multi-lineage differentiation potential after extensive expansion, compared to those of the static cultures.[10,11] Recent studies have focused on the effects of flow-induced shear stress on osteogenic differentiation and cell activity.[12-13] Several investigations have reported that extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun NH2-terminal kinase (JNK), and p38 members of the mitogen activated protein kinase (MAPK) family are important key regulators in osteogenic differentiation of MSCs induced by fluid shear stress (FSS).[14] Many upstream signaling molecules can phosphorylate ERK1/2 in the presence of physical force. Phosphorylated ERK1/2 (pERK1/2) can in turn upregulate and activate pre-growth transcription factors, such as cyclo-oxygenase (COX-2) and a cellular proto-oncogene (c-fos), as well as some cytoskeletal components like α-actinin.[15,16] Oscillatory fluid flow and steady fluid flow regimens enhance the expression of phenotypic markers of osteoblast cells such as OPN and OCN, and this effect appears to be related to the release of prostaglandin E_2 (PGE_2).[17] The expression of COX-2, the inducible isozyme for PGE_2 synthesis, is directly upregulated by mechanical loading through the formation of focal adhesions subsequent to ERK and protein kinase A (PKA) signaling pathways in osteoblasts.[17] Stimulation of mechanosensors by fluid flow results in intracellular events such as an increase in intracellular Ca^{2+} release synthesis, release of NO and PGE_2, and activation of MAPK.[18-20] Ion channels are the first described mechanosensors to respond directly to membrane perturbation.[21] Ca^{2+} channels are especially critical for the bone marrow mesenchymal stem cell (BMSC) response to FSS. Physical stimulation can also lead to the reorganization of the actin cytoskeleton.[22] The actin cytoskeleton links cell surface receptors such as integrins and proteoglycans to the internal architecture and plays a critical role in determining the mechanical properties and signaling pathways that regulate intracellular processes and protein expression. Matrix metalloproteinases (MMPs) are speculated to be relevant to mechanical signal transformation. MMPs can cleave the extracellular matrix (ECM) and substrate proteins to release signaling molecules such as transforming growth factors beta (TGF-β) and initiate signal transduction.[23,24] Physical forces on cells as a major mechanical stimulus also lead to an increase in the expression of MMPs in osteoblasts.[25] Focal adhesion kinase (FAK) is important for FSS-induced mechanotransduction in osteogenic differentiation.[26] Most cells persist in a state of dynamic flux where gases and diffusable molecules act either indirectly or directly as ligands that bind to the specific receptors on the cell surface, thereby modulating the cellular phenotype. Furthermore, cell-cell and cell-matrix interactions also play a critical role in modulating gene expression since the matrix and/or fluids surrounding the cells move, expand, or contract over the cell surface.[27,28] Shear forces act in a variety of ways. Firstly, the effects can be transmitted directly via transmembrane proteins connecting the cell's interior organelles. Secondly, cell signaling cascades can also lead to changes in gene expression.[29] Additionally, phenomena that may be influenced include both mechanoreception (e.g., plasma membrane receptors, ion channels, integrins/focal adhesions and protein kinase G (PKG) signaling) and cellular responses (e.g., intracellular Ca^{2+}, NO, PGI_2 and cytoskeletal remodeling).[30] There is evidence that mechanical inputs like hydrostatic pressure and fluid flow-induced shear force during *in vitro* culture may produce tissues with characteristics more like *in vivo* structures.[31,32]

3. Mechanotransduction of Physical Force in MSCs

The precise mechanism by which a physical force is translated into a chemical signal is not clearly understood, but appears to depend on the specific sensory molecules and physical structures involved. The surface of a cell contains transmembrane receptors, ion channels, integrins, cavoelae, glycocalyx components and primary cilia. The initial step of mechanotransduction is to detect the mechanical stimulation. Research on mechanotransduction has shown that gaps between physical forces and biochemical signals are bridged by ion channels such as K^+ and Ca^{2+} channels, intracellular signaling, inositol 1, 4, 5 triphosphate (IP_3), mechanically induced signaling molecules such as PGE_2, NO, and transmembrane molecules such as integrins and growth factors.[33,34] It has been accepted that multiple processes are involved in mechanotransduction of physical forces with various stimulators in MSCs; the response of cells to the physical stimulation, the transformation of mechanical signals into biochemical signals and the transduction of intracellular biochemical signals into the nucleus to regulate the expression of genes specific for osteogenic differentiation.[4] Physical forces on cells are transmitted through the ECM, which consists of tissue-specific proteins such as collagen, laminin and fibronectin and adhesion complexes at the cell surface, which physically link the ECM to the cytoskeleton, as shown in Fig l.

3.1 Ion Channels on Mechanotransduction

The cell membrane is a selectively permeable lipid bilayer coated by proteins that comprise the outer layer of a cell. The cell membrane exists between the machinery on the inside of the cell and the outside fluid that bathes the cells. Since the first observation that ion channels can be gated by mechanical strain, mechanotransduction has been proposed as a primary function of these channels. Moreover, mechanosensitive channels are likely candidates for the initial biochemical coupling mechanism of mechanical strain since no second messenger is required for channel activation. The flow of interstitial fluid through the lacuno-canalicular network induces shear stress on the membrane of bone cells. This FSS is a likely candidate for signal-to-bone cell adaptive responses.[35] Bone cells, in particular osteocytes, are extremely sensitive to mechanical stress rather than to streaming potentials mediated by the transport of ions with the flow. These cells were subjected to various levels of shear and changes in cellular responses. Patch-clamp technique, which was introduced by functional investigations of ion channels, has been used to show at least three classes of mechanosensitive ion channels in human osteoblasts.[35] Deformation of the cell membrane and alteration of membrane proteins induced by physical stimulation can cause stretch-activated ion channels to open and consequently lead to the influx of cations into the cells.[36] The release depends on L-type voltage-sensitive Ca^{2+} channels and mechanosensitive cation-selective channels induced by the influx of extracellular Ca^{2+}, which is the activator of the MAPK pathway.[37] Liu *et al.* (2008) reported that extracellular Ca^{2+} influx induced by physical forces has been implicated as an important regulator of integrin and F-actin cytoskeleton realignment.[37] In addition, mechanotransduction is also driven by G-protein coupled receptors (GPCRs).[37] It is well known that mechanical stress stimulates G protein activation. Activation of certain GPCRs activates PKA, which phosphorylates the cyclic adenosine monophosphate (cAMP) response element (CRE) that binds the cAMP response element-binding protein (CREB), a

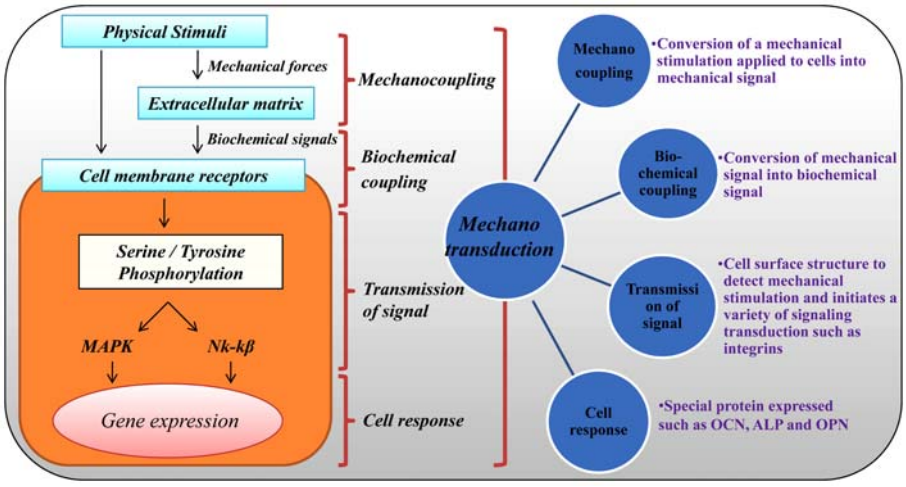

Figure 1. Mechanotransduction mechanism in osteogenic differentiation induced by physical stimulation.

transcription factor that can bind to the promoter of the COX-2 or the fosB/ΔfosB gene. The intracellular Ca^{2+} concentration increases in the very early cellular response of mechanotransduction.[38-40] Ion channels are one of the factors affecting cellular response to physical stimulation.[41]

3.2 Integrin Signaling

Forces concentrated in focal adhesion sites can then stimulate integrin dimerization and recruitment of focal adhesion proteins paxillin, talim, and vinculin which connect directly to microfilaments and indirectly to microtubules and intermediate filaments.[42] Focal adhesion sites between the cell and the ECM function as mechanosensors that connect mechanical signals of physical stimulation from the ECM with cytoplasmic signaling molecules in a mechanism referred to as mechanotransduction in osteoblasts.[43,44] Integrins are cell adhesion transmembrane molecules that serve as receptors for ECM proteins. Experimental evidence supports the idea that external stimuli activate intracellular focal adhesion components.[45] In the case of FSS, this external stimulation induces integrin-Shc association, depolarization of the membrane, and increased phosphorylation of FAK. Integrins are composed of two subunits, denoted as α and β, both of which are required for cell adhesion.[46] These subunits can be interchanged, permitting different binding specificities for different ECM proteins. The cytoplasmic tail of the integrins, which is the β subunit, has been shown to have a critical role in integrin signaling.[47] Integrin signaling pathways are positioned to transduce physical forces into chemical signals.[48] β1 integrins play a prominent role in shear-induced signal conduction and bone formation-related gene expression in osteoblast-like cells.[49] Integrin αvβ3 is also activated in response to fluid flow, as shown by the recruitment of the adaptor molecule, Shc.[50,51] Fredrick *et al*. (1998) indicated that Ras homolog gene (RhoA)-dependent stress fiber formation in response to physical forces is involved in the mechanically induced upregulation of COX-2 and c-fos in osteoblasts.[52] It appears that integrin signaling leads to alterations in Rho stress fiber formation and phospholipase C (PLC) activation. PLC generates IP_3 which is used for signal transduction in cells and ultimately increases intracellular Ca^{2+} levels. Integrin α5β1 has been shown to have an important role in the regulation of MSCs osteogenic differentiation in two-dimensional and three-dimensional cultures.[53,54] Physical forces like flow shear stress have been shown to activate MAPK via β1 integrin and FAK-dependent tyrosine phosphorylation. Integrin binding leads to activation of FAK in response to extracellular stimuli and during cell adhesion to ECM ligands. Inhibition of FAK blocks the transcriptional activity and the osteogenic differentiation of human mesenchymal stem cells (hMSCs). Upon phosphorylation, FAK contributes to MAPK activation via interaction with Shc, Grb2, small GTPase, and Rat sarcoma (Ras).[56-59] This is significant as MAPK activation induces osteogenic differentiation. These signals in osteoblasts serve to activate MAPK signaling initially via extracellular signal-regulated kinase (MEK), which ultimately culminates in the phosphorylation of ERK1/2. ERK1/2 mediates osteoblast differentiation through activation of Runx2. [60]

3.3 Rho Kinase Signaling

The activated Rho has an additive effect on Runx2 expression. The Rho signaling pathway affects actin cytoskeleton organization. The cytoskeleton plays a key role not only in cell-shape stability and migration, but also in signaling pathways that regulate intracellular processes and protein expression in response to a biomechanical environment.[61,62] The contraction of actin-cytoskeleton is critical for the differentiation of hMSCs. Therefore, the over-expression of Rho and Rho-associated kinases (ROCKs) promote the osteogenic differentiation of hMSCs. RhoA regulates the formation of contractile actin myosin filaments to form stress fibers. Roles of the cytoskeleton as a mechanotransducer have been articulated in studies of cellular responses to changes in substrate stiffness and cell shape, stretching of cells, and shear stress. The cytoskeleton is rearranged, and the amount of special structural proteins in the cytoskeleton changes in response to physical stimulation.[63,64] Several studies have concluded that fluid shear-induced mechanical signaling in osteoblasts leads to increased expression of COX-2 and c-foc through a mechanism that involves reorganization of the actin cytoskeleton. Thus, Rho-mediated stress fiber formation and the α-actinin-dependent anchorage of stress fibers to integrins in focal adhesions may promote fluid shear-induced metabolic changes in bone cells. RhoA effects actin-cytoskeleton organization through the activation of ROCKs.[63,64] Rho proteins are active when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP). When membrane fluidity is greatly increased with the use of lysophosphatidylcholine, mechanical stress caused an increase in GTPase activity.[33] This demonstrates the ability of the phospholipid bilayer to mediate shear stress-induced activation of GTPase and also shows that the physical properties of the bilayer modulate mechanotransduction.[49] The upstream signals regulating Rho GTPase activity, such as integrin and TGF-β, may induce the relocalization of gunaine nucleotide exchange factors (GEFs) to membrane structures containing the GTPase targets. GTP replaces GDP to activate Rho and GEFs are released from Rho-GTP.[65] Activated

Rho interacts with ROCKs and phosphorylates myosin phosphatase. Activated myosin phosphatase activates myosin light chain to increase the contraction of actin-cytoskeleton.[66]

3.4 NO and G-Protein Signaling

NO has been shown to be essential for adaptive bone formation *in vivo*.[67, 68] Bone cells are more responsive (in terms of NO and PGE_2 production) to shear stress by fluid flow stimulation. Several studies have shown that NO plays an important role in the osteogenic differentiation.[69] McGarry *et al*. (2005) found that NO response induced by the fluid flow in osteoblasts was accompanied by parallel alignment of stress fibers, whereas PGE_2 response was related to fluid flow stimulation of focal adhesions formed after cytoskeleton disruption.[70] Increased intracellular Ca^{2+} leads to activation of constitutively expressed NO synthases, and increased NO production in osteoblasts occurs with flow onset.[71-73] Stress-induced expression of c-fos, fra-1, fra-2 and fosB/ΔfosB mRNAs is dependent on signaling through the MEK/ERK pathway. This increases the activity of transcription factors such as Runx2 and AP-1.[73] The sustained stimulation of NO synthesis (NOs) activity may occur through endothelial nitric oxide synthesis (eNOS) phosphorylation by multiple kinases as observed in stressed endothelial cells.[74] NOs-deficient mice demonstrate reduced post-natal bone mass because of defects in osteoblast number and maturation, and impaired bone formation in response to mechanical stimulation of cAMP response element-binding, which is a cellular transcription factor.[75,76] In the event of unstimulated cells, Serine (Ser) is not phosphorylated but it is rapidly phosphorylated after the application of mechanical stimulation.[77] NO activates soluble guanylate cyclase (sGC), which is activated upon NO binding to the heme prosthetic group of the enzyme, resulting in increased cGMP. The intracellular cGMP concentration activates PKG, based on increased vasodilator stimulated phosphoprotein (VASP) phosphorylation on Ser, a site targeted by both PKG I and PKG II.[77] The activation of these transcription factors promotes the transcription of Col-I, OCN and other special genes involved in osteogenic differentiation.[78] PGE_2 is an integral messenger in mechanochemical signal transduction and has been shown to regulate bone formation both *in vivo* and *in vitro*. Physical stimuli on cells can induce GTP-binding protein (G protein). G proteins and Ca^{2+} influx play an important role in the flow-induced PGE_2 production in osteoblasts.[79] All PGE_2 receptors are GPCRs that undergo conformational changes in response to increased plasma membrane tension. More specifically, pectenotoxins (PTX)-sensitive G proteins appear to be responsible for the majority of the flow-induced PG production in osteoblasts.[80] However, only the initial burst of NO synthesis is calcium dependent, whereas the later sustained phase of NO production is calcium independent. The binding of PGE_2 to its receptor leads to the activation of adenyl cyclase (AC), which converts adenosine-5'-triphosphate (ATP) into cAMP and activates cAMP-dependent PKA.[81] PKA elicits an immediate response through the induction of genes such as those encoding inhibitors of DNA binding proteins and fos-related antigen (fosB), followed by sustained secretion of bone-related cytokines such as bone morphogenetic protein (BMP) 2, insulin-like growth factor 1 (IGF-1), and interleukin 1 (IL-1). Eventually, physical stimulation on cells increases the TGF-β1 mRNA expression and TGF-β1 protein production in osteoblast-like cells. Two amino acids, Ser residue in the reductase domain and a threonine residue (Thr) located within the CaM binding domain, seem to be particularly important in regulating eNOS activity.[82-84] These bone-related cytokines can up-regulate the expression of osteogenic differentiation-related genes, such as ALP and Col-I.[85]

3.5 Calcium Signaling

Mechanical stimulation on cells results in mobilization of the intracellular calcium concentration in hMSCs. An enhanced concentration of Ca^{2+} caused by physical forces can activate the Ca^{2+} signaling pathway. A major source Ca^{2+} can bind calmodulin (CaM) to form the calcium/calmodulin complex and initiate the activation of the Ca^{2+} signal pathway, which leads to the activation of ERK1/2.[86] pERK1/2 can activate many transcription factors, including AP-1 family transcription factors such as ΔfosB and fosB.[87] FosB belongs to the AP-1 family of transcription factors, a group of proteins known to regulate osteogenic differentiation and bone formation. FosB may indirectly contribute to enhanced osteogenic differentiation via generation of ΔfosB. Splicing of fosB to produce ΔfosB may occur at a fixed rate, and the more fosB is expressed, the more it gets spliced. Alternatively, physical stimulation may increase the amount of ΔfosB relative to fosB, ultimately leading to improved osteoblast function.[87,88] The AP-1 family of transcription factors bind a consensus sequence in the promoters of several genes that are essential for osteogenic differentiation such as OPN, OCN and Runx2. Mechanical loading by physical forces on cells induces fosB transcription, which is caused by the activation of ERK1/2 by the calcium pathway. Ca^{2+} is one of the most important biological signals. It has been proven that Ca^{2+} plays an important role in mechanotransduction. It is well established that Ca^{2+} is regulator of transcriptional changes in gene

expression, which affects the transcription of numerous phenotypic genes and transcription factors that are important for cellular function, proliferation, and differentiation.[89, 90]

4. MAPK Signaling Pathway

MAPK cascades play a central role in cell proliferation and differentiation by transmitting signals from the cell membrane to the nucleus. In mammals, there are more than a dozen MAPK genes. The best-known MAPK genes are the ERK1/2, JNK (1–3) and p38 (α, β, and Y) families. ERK3, ERK5 and ERK7 are other MAPKs that have distinct regulation and functions.[91] ERK1/2 and p38 have been most widely investigated in FSS-induced mechanotransduction in hMSCs. MAPK cascades consist of a core of three protein kinases.[92] Specifically, the common target of MAPKs induced by mechanical stress during osteoblast differentiation is believed to be the ERK pathway, which is mediated by ATP-dependent Ca^{2+} influx at calcium channels via PKC, GPCRs, and PKA activation. In addition, the high expression of integrins and the activation of FAK promote the activation of Ras adaptor proteins.[60,93] Activated Ras initiates MAPK signaling cascades by activating Raf, leading to the activation and thus phosphorylation, of the MEK and ERK/JNK MAPK.[93] JNK and ERK target an overlapping set of transcription factors resulting in the differential activation of immediate early gene products that belong primarily to the Jun (c-Jun, JunB, JunD) and Fos (c-fos, fosB, fra-1, fra-2) families. The Fos family of proteins, together with c-Jun, are the major components of the transcription factor AP-1, which is pivotal in controlling gene expression, especially in the early stages of osteoblast differentiation.[94] Several studies have demonstrated that the expression of the major JNK/ERK substrate and the transcription factor AP-1 increases under the influence of mechanical forces. This signaling cascade is important to bone and cartilage load-ignited osteogenic differentiation. ERK1/2 is involved in cell transformation, proliferation, and survival, whereas p38 participates in many cellular processes such as cell cycle control, inflammatory response, apoptosis, and differentiation of cells including osteoblasts.[94] The p38 signaling pathway in hMSCs can be activated by cell stimulation, but p38 has a non-essential role in stress mechanotransduction.[95] Fig 2 shows the hypothetical intracellular signaling pathway from a cell-surface receptor to the nucleus. A series of signaling proteins and small intracellular mediators relay the extracellular signal into the nucleus, causing a change in gene expression.

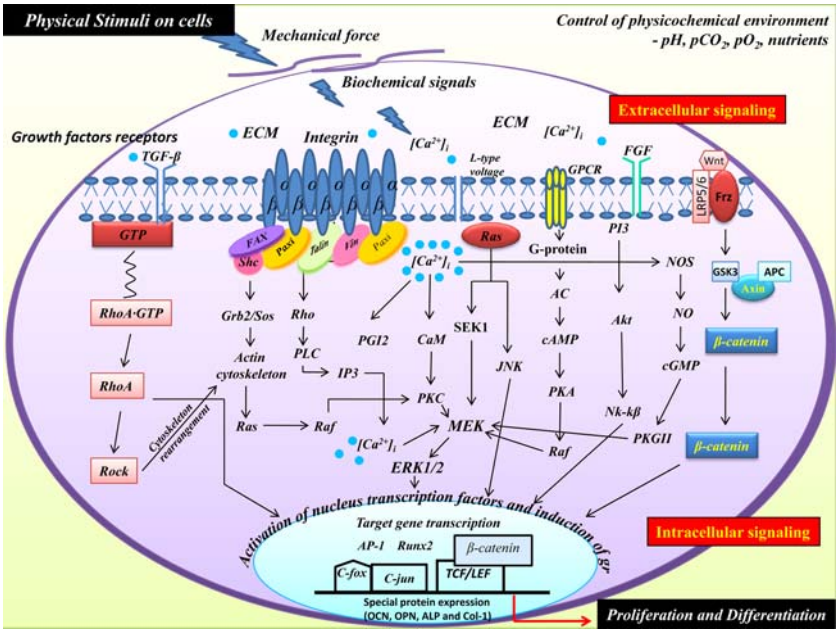

Figure 2. Hypothetical molecular signaling pathways from ECM to the nucleus. Physical stimulation on MSCs activates stretch-activated ion channels and cell surface receptors such as integrin, GPCR and LRP5/6 bound to the ECM. Stretch activated ion channels allow extracellular Ca^{2+}, and indirectly Na^{+}, to flow in and activate Ca^{2+} responsive proteins such as PKC, CaM kinase and calcineurin. PKC and CaM kinase activate the transcription factors, AP-1 and Runx2. Integrin receptors recruit adaptors and cause three main changes: (1) the phosphorylation and activation of MAPK signaling pathways (MEKK and JNK) that lead to ERK1/2 phosphorylation, (2) the activation of PLC leading to IP_3 generation and gating of intracellular Ca^{2+} stores, and (3) rearrangement of the actin cytoskeleton. pERK1/2 promotes the activation of the AP-1 and Runx2 transcription factor families, which are necessary for bone remodeling. Activated AP-1 and Runx2 transcription factors upregulate osteogenic differentiation-related expression of genes such as OCN, OPN, ALP and Col-I.

The signal is altered, amplified, distributed, and modulated. Because many of the steps can be affected by other extracellular and intracellular signals, the final effect of one extracellular signal depends on multiple factors affecting the cell. Ultimately, the signaling pathway activates effector proteins that alter cell behavior.

5. Wnt Signaling Pathway

Wnt glycoproteins transduce multiple signaling cascades including the canonical Wnt/β-catenin, the noncanonical Wnt/non, the Wnt/Ca^{2+}, and Wnt/polarity pathway.[96] LRP5 is a single-pass transmembrane protein that functions as a co-receptor for the secreted family of Wnt glycoproteins.[97] β-catenin, one of the targets of GSK-3β, acts as a transcriptional co-activator of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) protein. Flow-induced β-catenin signaling may be regulated by adherent junctions in MSCs. Oscillatory fluid flow induces β-catenin nuclear translocation and the initiation of TCF/LEF/β-catenin transcription, which is necessary for flow-induced Runx2/Cbfa1 expression.[97] Especially, the canonical Wnt/β-catenin signaling pathway has been implicated in the promotion of bone formation. Wnt proteins bind to the frizzled (Frz) transmembrane receptors and low density LRP5/6 coreceptors located on the cell surface to form a receptor trimeric complex, resulting in the inhibition of glycogen synthase kinase-3β (GSK-3β).[98] Mechanical stimuli on cells are a powerful tool that initiates both noncanonical Wnt signaling and canonical Wnt/â-catenin signaling. Many studies have demonstrated that lipoprotein receptor-related protein 5/6 (LRP5/6) and the canonical Wnt/β-catenin signaling pathway are essentially required for bone formation.[99] Canonical Wnt/β-catenin induces the early osteoblast differentiation marker ALP in MSCs.[100,101] Basically, it plays an important role in bone cell proliferation, differentiation, and inhibits apoptosis.[102] The canonical Wnt/β-catenin pathway has been shown to induce ALP activity in osteogenic cultures of the mouse cell lines[103] However, the precise role of Wnt signaling in bone biology still remains unclear although this pathway plays a pivotal role in the differentiation of MSCs along either of these two lineages, promoting osteogenesis and inhibiting adipogenesis.

6. Osteoblast-Related Transcription Factors Runx2 and AP-1

Runx2 and AP-1 are essential transcription factors for osteoblast differentiation and bone formation. As mentioned above, defining the molecular mechanisms by which Runx2 can function as a master regulatory gene for activating the program of osteoblast genesis has provided novel insights for transcriptional regulation of tissue-specific genes. Regulation of Runx expression has the potential to serve as a basis for the design of novel therapeutic strategies for promoting bone formation.[104] The expression and functional activity of Runx2 and AP-1 are controlled by ECM and intracellular signaling pathways including the ERK/MAPK pathways (ERK1/2 and JNK, not p38). Above all things, Runx2 is a key target of mechanical signals that mediate several key functions for regulating skeletogenesis, controlling osteoblast growth and differentiation, and integrating the complex pathways required for bone formation. There is also considerable evidence that the Runx family of transcription factors is required for the activity of TGF-β-related factors, including bone matrix proteins (BMPs).[105] TGF-β1 has been shown in multiple studies to stimulate bone formation. AP-1 transcription factor is a dimeric protein complex that is composed of Jun (c-Jun, JunB, JunD) and Fos (c-fos, fosB, ΔfosB, fra-1, fra-2) proteins. C-jun and c-fos expression are important to osteogenic differentiation. COX-2 is necessary for mechanically induced bone formation. AP-1 transcription factor is strongly induced in response to numerous signals including growth factors, cytokines, and extracellular stresses. These proteins are functionally related to osteoblast maturation. Physical stimulation eventually enhances the dimeric protein's activity in MSCs.[105] These Runx2 and AP-1 transcription factors are regulators of osteoblast phenotype genes such as OCN, OPN, Col-I, and bone sialoprotein (BSP) in the response of MSCs to physical stimulation.[105,106] Finally, molecular responses in osteogenic differentiation by various physical stimulations were summarized, as shown in Table 1.

7. Summary and Conclusions

The differentiation of MSCs into osteoblasts is promoted by physical stimulation through mechanotransduction mechanisms including extracellular and intracellular signaling pathways. A number of studies have focused on how physical stimulation alters the activities of multiple molecules at various sites in the cytoplasm and nucleus in order to have therapeutic applications in bone-related diseases and bone regeneration. The process involves multiple signaling pathways such as integrin, calcium, NO, PGE_2, and G-protein signaling pathways which are activators of the AP-1 and Runx2 transcription factors. The activation of Runx2 and AP-1 is essential for osteogenic differentiation and bone regeneration. In this review, we provide information about the molecular mechanisms

Table 1. Molecular responses in osteogenic differentiation by physical stimulation.

Cell types	Functions	Roles	Physical stimulation apparatus	References
hMSCs	Up-regulates gene expression of MAP3K8 and IL1B	IL1 beta is a cytokine that is released in response to a variety of cellular stresses	Streamer fluid shear bioreactor	John *et al*., 2009.[14]
MC3T3-E1 cells	Cytoskeleton reorganization and increases in c-fos and COX-2 transcript	C-fos is required for osteoblast cell cycle control	Parallel plate flow chamber	Chen *et al*., 2000.[18]
MC3T3-E1 cells	Induction of COX-2 expression is via a PKA, not PKC signaling pathway	COX-2 is a subunit of the AP-1. transcription factor	Parallel plate fluid flow chamber	Wadhwa *et al*., 2002. [20]
MC3T3-E1 cells	ERK1/2 phosphorylation requires Ca^{2+}-dependent ATP release, which is mediated through P2Y7	P2Y7 and P2Y2 are P2 receptors	Perfusion flow bioreactor	Liu *et al*., 2008.[37]
Human osteoblast-like cells	Up-regulation of interleukin-11 mediated by a prostaglandin-mediated pathway	IL-11 is an important regulator in bone metabolism	Cone-plate viscometer apparatus	Sakai *et al*., 1999. [41]
MC3T3-E1 cells	Expression of COX-2 and increases the release of PGE_2	COX-2 is necessary for mechanically induced bone formation	Parallel plate flow chamber	Ponik *et al*., 2004.[45]
MC3T3-E1 cells	c-fos, fra-1, fra-2 and fosB/fosB mRNA expression in osteoblasts via NO/cGMP/PKG II and through MEK/ERK, but not the p38 MAPK pathway.	ERK is essential for osteoblast growth and differentiation	Parallel plate flow chamber	Rangaswami *et al*., 2009.[73]
C3H10T1/2 progenitor cells	OFF regulates osteogenic differentiation via the activation of RhoA/ROCKs II and ultimately isometric tension in the actin cytoskeleton.	RhoA has the potential to regulate Runx2 expression.	Parallel plate flow chamber	Emily *et al*., 2009.[80]
Murine primary osteoblasts cells	Activation between PKC-Smads and fosB/JunD pathways to synergistically stimulate IL-11 gene transcription.	IL-11 is a bone-related cytokine.	Horizontal rotating platform apparatus	Kido *et al*., 2010.[88]

underlying osteogenic differentiation in MSCs that are induced into the osteogenic lineage pathway by physical stimulation. Our hope is to facilitate the identification of new approaches for the treatment of bone-related diseases and bone regeneration. Finally, based on our review, we propose the following directions for future research:

(1) In-depth studies of the various physical stimulation effects, including the signaling pathways activated for osteogenic differentiation of MSCs.

(2) Analysis of the relationship between physical stimuli and the matrix in osteogenic differentiation of MSCs.

(3) Investigation of the effects of combining scaffolds, MSCs, physical stimuli, and biochemical stimuli on osteogenic differentiation of MSCs.

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