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REVIEW

Mechanisms for osteogenic differentiation of human mesenchymal stem cells induced by fluid shear stress

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Abstract Mechanical stimuli can improve bone function by promoting the proliferation and differentiation of bone cells and osteoblasts. As precursors of osteoblasts, human mesenchymal stem cells (hMSCs) are sensitive to mechanical stimuli. In recent years, fluid shear stress (FSS) has been widely used as a method of mechanical stimulation in bone tissue engineering to induce the osteogenic differentiation of hMSCs. However, the mechanism of this differentiation is not completely clear. Several signaling pathways are involved in the mechanotransduction of hMSCs responding to FSS, such as MAPK, NO/cGMP/PKG and Ca^{2+} signaling pathway. Here, we briefly review how hMSCs respond to fluid flow stimuli and focus on the signal molecules involved in this mechanotransduction.

Keywords FSS · hMSC · Integrin · Glycocalyx · MMPs · NO · PGE2 · FAK · MAPK · Ca^{2+}

1 Introduction

Bone in a healthy person or animal can adapt to mechanical loads (Wolff 1986). If the loading on a particular bone is increased, the bone may be remodeled and become stronger to resist the loading. An appropriate mechanical loading is necessary for bone to maintain homeostasis (Harada and Rodan 2003). The mineral content of an astronaut's bone decreases due to the microgravity during spaceflight (Zayzafoon et al. 2004), and bone hypertrophy occurs when bone is subjected to increased mechanical loads (Tschantz and Rutishauser 1967; Turner et al. 1994). Various mechanical stimuli including compressive, tensile, curvature, torsional and fluid shear stress may produce the loading effects on bone.

Bone formation in vivo, no matter it stems from embryonic development, normal growth, remodeling or fracture healing, follows a common course. It begins with the aggregation of undifferentiated mesenchymal cells or preosteoblasts followed by a period of proliferation to provide sufficient cell numbers for the tissue. After the initial cell proliferation, the cells start to synthesize and secrete a loose organic matrix and differentiate into osteoblasts. Mesenchymal stem cells (MSCs), as precursors of osteoblasts, exist in bone marrow where there is a unique mechanical environment. The main mechanical forces in this environment are hydrostatic pressure and fluid shear stress (FSS). FSS, as a major stress produced by the interstitial fluid flow (IFF) through the lacunar or canalicular spaces and exerted on the cell surface, can generate the mechanical stimulus to cells, and then this stimulus is translated into biochemical signals in cells to exert biological effects, such as affecting the proliferation of osteocytes and the differentiation of osteoblasts and mesenchymal stem cells. IFF can be driven by mechanical loading and bone bending that cause strain gradients as well as local pressure gradients in the bone and in the medullary cavity (Gurkan and Akkus 2008). The level of FSS induced by IFF in lacuna-canalicular spaces within bone tissue is about 8–30 dyn/cm^2 and it may vary due to physical activities (Weinbaum et al. 1994). FSS is a significant stimulus for osteoprogenitor cells (such as MSCs) in the marrow and results in recruitment, proliferation and osteogenic differentiation of these cells in bone formation sites (Li et al. 2004). In recent years, human MSCs (hMSCs) have been widely used as the seeded cells to construct the engineered bone tissue. An ideal engineered bone tissue should have

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the capacity to adapt to its functional environment and meet the mechanical demand. Therefore, in order to construct the ideal artificial bone tissues with properties of natural bones, FSS is also used in bone tissue engineering to simulate the environment of mechanical stimuli in vivo and promote the formation of artificial bones (Bjerre et al. 2008; Martino et al. 2009; Marolt et al. 2006; Yang et al. 2010).

Bioreactors are wildly used to produce FSS in bone tissue engineering, especially in the bone tissue engineering with hMSCs as seeded cells. A great deal of research has demonstrated that FSS enhances deposition of mineralized matrix and expression of osteoblastic specific genes in hMSCs (Bancroft et al. 2002; Kreke et al. 2005; Scaglione et al. 2008; Sharp et al. 2009; Zhao et al. 2007). It has been accepted that three processes are involved in mechanotransduction of FSS in hMSCs: the response of cells to the physical stimulus (mechanocoupling), the transformation of mechanical signal into biochemical signal (biochemical coupling) and the transduction of intracellular biochemical signal into the nucleus to regulate the expression of genes specific for osteogenic differentiation (see Table 1) (Duncan and Turner 1995).

hMSCs respond to FSS via the recognition of cell surface receptors to the mechanical stimulation. Ion channels and integrins are considered as mechanoreceptors involved in mechanotransduction. Ion channels are the first described mechanosensors to respond directly to membrane perturbation (Guharay and Sachs 1984). Ca^{2+} channels are especially critical for hMSC's responses to FSS. FSS can induce a high concentration of Ca^{2+} in hMSCs (Riddle et al. 2006; Stiehler et al. 2009). In addition, integrins are transmembrane receptor proteins that connect cytoskeleton with extracellular matrix (ECM) and are also considered as main mechanoreceptors (Kamioka and Yamashiro 2008; Lee et al. 2008). Moreover, the surface proteoglycan layer, glycocalyx, is also considered as a primary mechanotransducer for mechanical stimuli. It has been demonstrated that glycocalyx is important for the mechanotransduction of FSS in some types of cells, such as endothelial cells and leukocytes (Coughlin and Schmid-Schonbein 2004; Kawano et al. 2002). FSS can also lead to the reorganization of actin cytoskeleton (Patwari and Lee 2008). It was reported that actin cytoskeleton in hMSCs was remolded during osteogenic differentiation (Titushkin and Cho 2007). Actin cytoskeleton links cell surface receptors, such as integrins and proteoglycans, to internal architecture and plays a pivotal role in determining the mechanical properties and signaling pathways that regulate intracellular processes and protein expression. Therefore, the remodeling of actin cytoskeleton may also play an important role in the mechanotransduction of FSS in hMSCs.

Matrix metalloproteases (MMPs) are speculated to be relevant to mechanical signal transformation. Mechanical stimuli can regulate the expression of MMP gene family and break the balance of MMP/TIMP (tissue-specific inhibitors of metalloproteases) in hMSCs (Kasper et al. 2007; Fehrenbacher et al. 2003; Sternlicht and Werb 2001). MMPs can cleave ECM and substrate proteins to release signaling molecules, such as transforming growth factor β (TGF-β), and initiate signal transduction. FSS, as a major mechanical stimulus, also leads to an increase in expression of MMPs in osteoblasts (Charoonpatrapong-Panyayong et al. 2007).

In recent years, more studies have focused on the intracellular signal transduction rather than the extracellular responses. Focal adhesion kinase (FAK) plays an important role in the mechanotransduction of FSS (Goessler et al. 2008). FSS also increases the level of nitric oxide (NO) and prostaglandin E2 (PGE2) (McAllister et al. 2000). NO acts as the second messenger to activate the PKG signaling pathway. Furthermore, an enhanced concentration of Ca^{2+} caused by FSS can activate the Ca^{2+} signaling pathway. Through these signaling cascades, FSS finally activates extracellular signal-regulated kinase1/2 (ERK1/2), which can influence the expression and activation of transcriptional factors, such as runt-related transcription factor 2 (Runx2) and activator protein1 (AP-1) (Grellier et al. 2009; Rubin et al. 2006). These transcription factors can subsequently initiate the transcription of specific genes related to osteogenic differentiation, such as alkaline phosphatase (ALP), osteocalcin, collagen I and osteopontin. With the expression of osteogenic differentiation-related genes, hMSCs differentiate into osteoblasts.

This review is focused on mechanoreceptors, mechanosensors, intracellular signaling molecules and signaling pathways that play essential roles in the mechanotransduction of FSS in hMSCs.

2 Detection of mechanical stimulation caused by FSS

The primary step of mechanotransduction is to detect mechanical stimulus. Ion channels, integrins, glycocalyx and cytoskeleton are very important for mechanically sensitive cells to detect FSS (Liu et al. 2008; Salter et al. 2001; Tarbell et al. 2005). Via above cellular elements, mechanical signals can be transmitted to apical structures of cells such as the plasma membrane or the actin cortical web, where transduction usually occurs or signals are transmitted to the intracellular regions of the cell (Tarbell et al. 2005).

2.1 Ion channel

FSS-induced deformation of cell membrane and alteration of membrane proteins can cause stretch-activated ion channels to open and consequently lead to the influx of cations, such as Ca^{2+}, Na^{+} and K^{+}, into the cell (McMahon et al. 2008). Intracellular Ca^{2+} concentration increases by tenfold in 3-D dynamic cultured hMSCs after FSS stimulation (Stiehler et al. 2009). However, Ca^{2+} release in

Table 1 Definition of special terms

	Definition	References
Mechanotransduction	The process of mechanical signal leading to cell response including four distinct steps: mechanocoupling, biochemical coupling, transmission of signal and cell response	(Duncan and Turner 1995)
Mechanocoupling	Conversion of a mechanical stimulation applied to cells into mechanical signal	(Robling et al. 2001; Duncan and Turner 1995)
Biochemical coupling	Conversion of mechanical signal into a biochemical signal	(Robling et al. 2001; Turner and Pavalko 1998)
Mechanoreceptor	Cell surface structure to detect mechanical stimulus and initiates a variety of signaling transduction, such as integrins	(Ali and Schumacker 2002)
Mechanotransducer/ mechanosensor	Cell structure or molecules to sense or transmit mechanical signals, such as ion channel, glycocalyx and cytoskeleton	(Tarbell et al. 2006; Sadoshima et al. 1992)
Special genes related to osteogenic differentiation	Special protein expressed when preosteoblasts differentiated into steoblasts so that cells have special properties of osteoblasts. Such as osteocalcin, ALP and osteopontin	(Catelas et al. 2006; Chen et al. 2008)

hMSCs is different from that in osteoblasts. In MC3T3-E1 osteoblasts, the inhibition of both L-type voltage-sensitive Ca^{2+} channel and the mechanosensitive cation-selective channel blocks the phosphorylation of ERK1/2 induced by FSS (Liu et al. 2008). In contrast, Riddle et al. (2006) reported that treatment of hMSCs with pharmacological antagonists against these channels had no significant effect on either fluid flow-induced Ca^{2+}level or ERK1/2 phosphorylation. As undifferentiated MSCs are non-excitable cells, only a small fraction of MSCs (15%) express functional L-type Ca^{2+} channel (Heubach et al. 2004). A major source of Ca^{2+} for the oscillations is the intracellular Ca^{2+} storage in hMSCs. The release of Ca^{2+} is mediated by inositol 1,4,5-trisphosphate receptors (InsP3Rs), whereas the entry of Ca^{2+} into cells through plasma membrane is mainly mediated by the store-operated Ca^{2+} channels (SOCs) with little contribution of voltage-operated Ca^{2+}currents (VOCCs) (Kawano et al. 2002). Therefore, compared to osteoblasts, hMSCs have a different way of releasing Ca^{2+} under FSS. Ca^{2+} can bind calmodulin (CaM) to form the Ca^{2+}/CaM complex and initiate the activation of Ca^{2+} signal pathway, which leads to the activation of transcription factors such as $\Delta FosB$ and FosB (both belong to AP-1 family transcription factors) associated with osteogenic differentiation. By summarizing the above discussion, ion channels are judged as important mechanosensors for the mechanotransduction of FSS in hMSCs.

2.2 Integrin and integrin-associated kinase

Many types of cells, such as osteoblasts, osteocytes, endothelial cells and hMSCs, are sensitive to mechanical stimuli, including FSS. FSS leads to similar outcomes in these cells. Therefore, it is possible that common mechanisms are involved in response to FSS. FSS is reported to induce the activation of ERK1/2 in osteobalsts, endothelial cells and hMSCs (Kim et al. 2007; Liu et al. 2008; Sumpio et al. 2005). Phosphorylated ERK1/2 can, in turn, up-regulate and activate transcription factors, such as AP-1 (Haasper et al. 2008; Lee et al. 2008; Nagel et al. 1999). Integrins, as the upstream molecules of mechanotransduction, in concert with other signaling molecules to activate ERK 1/2, are widely considered as mechanoreceptors in the mechanotransduction of FSS (Kapur et al. 2003; Ward et al. 2007; Weyts et al. 2002).

Integrins of focal adhesion complexes are heterodimeric transmembrane proteins that interlink ECM with cytoskeleton through several actin-associated proteins, including α-actin, vinculin, talin and tensin (Fig. 1) (Hynes 2002). Two subunits of integrin, α and β subunits, are non-covalently associated (Humphries 2000). To date, 18 different α subunits and 8 different β subunits have been described in vertebrates. These subunits form 24 distinct heterodimers, 12 of which contain the $\beta 1$ subunit (Al-Jamal and Harrison 2008). The main function of integrins is to mediate cell adhesion, link cells to ECM, make transmembrane connections to cytoskeleton, and activate many intracellular signaling pathways including mechanical signaling pathways (Hynes 2002). $\beta 1$ integrins play a prominent role in shear-induced signaling conduction and bone formation-related gene expression in osteoblast-like MG63 cells, such as fibronectin, type I collagen and laminin. Integrin $\alpha_V\beta_3$ also plays significant roles in such responses in osteoblasts (Lee et al. 2008). Therefore, integrins have an intimate relationship with mechanical stimulation because of their connection to ECM. Through this connection, integrins may detect the mechanical stimulation when cells are subjected to FSS. Integrin $\beta 1$/Shc (Shc, an adaptor protein containing C-terminal Src homology domain-2 (SH2) domain) association leads to the activation of ERK, which is critical for shear-induction of bone formation-related genes in osteoblast-like cells (Kapur et al. 2003; Lee et al. 2008). Similarly, integrins are also very

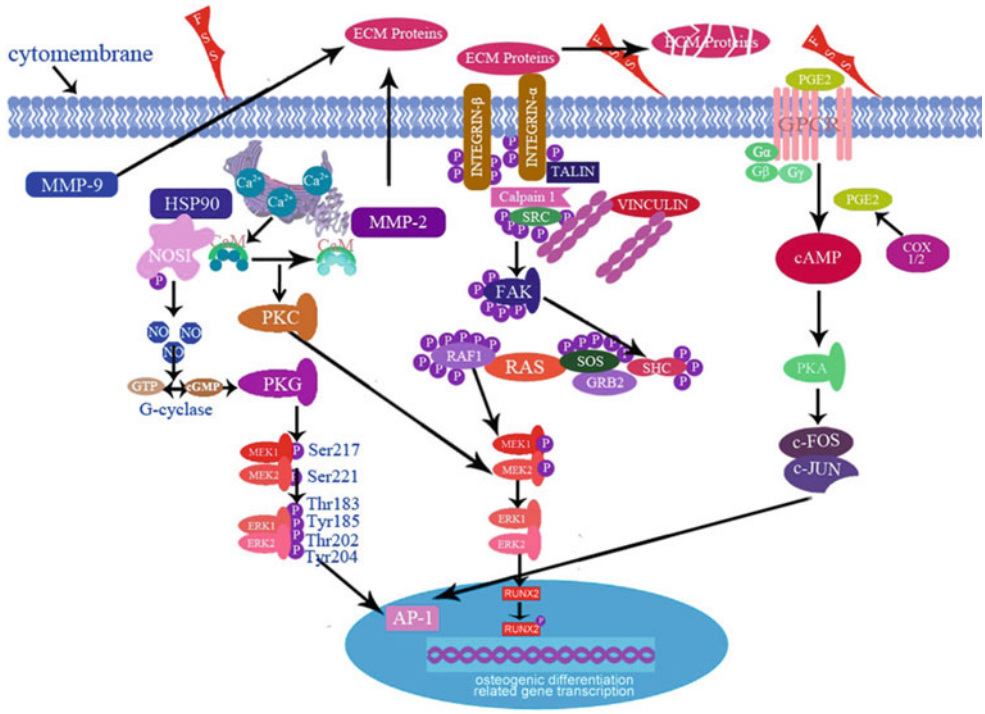

Fig. 1 Signal pathways involved in the osteogenic differentiation of hMSCs under FSS. FSS can activate many signal pathways in hMSCs. Integrins, connecting with cytoskeleton and ECM, act as important mechanical sensor for stimulation of FSS and participate in the formation of FA (Lee et al. 2008). Then integrin/FAK pathway activates the ERK1/2/MAPK signaling pathway (Kapur et al. 2003). NO and Ca^{2+} are important signal molecules in this mechanotransduction (McAllister et al. 2000). They can activate ERK1/2/MAPK signal pathway through PKG or PKC pathway. ERK1/2 seems to be the centre of this mechanism. Phosphorylated ERK1/2 can activate transcription factors, such as Runx2 and AP-1 (Damoulis et al. 2007; Rangaswami et al. 2009). Higher level of PGE2 caused by FSS can activate PKA signal pathway (Cherian et al. 2003). Activated ERK1/2 and PKA can activate transcription factors, such as Runx2 and AP-1, which can promote the transcription of osteogenic differentiation special genes (Kleiveland et al. 2008)

important in the osteogenic differentiation of hMSCs. FSS can up-regulate the level of $\alpha 5\beta 1$ in human adipose-derived MSCs (McIlhenny et al. 2010). Meanwhile, integrin $\alpha 5\beta 1$ has been demonstrated to have an important role in the regulation of MSC osteogenic differentiation in 2D and 3D culture (Martino et al. 2009). Therefore, it is inferred that the increased level of integrins promoted osteoblastic differentiation of hMSCs.

Integral membrane proteins laterally assemble into micrometer-scale clusters known as "focal adhesions" (FAs) (Hynes 2002). FAs recruit multiple kinase and phosphatase proteins, in addition to cytoskeletal proteins (Zhao et al. 2009). Several studies seem to support the concept that focal adhesions are sites of mechanotransduction. Blockade of focal adhesion formation decreases the production of cyclooxygenase-2 (COX-2) and PGE2. COX-2 and PGE2 are necessary for mechanical induction of bone formation (Li et al. 2002; Ponik and Pavalko 2004; Young et al. 2009). Focal adhesion kinase (FAK) responds to integrin clustering and FA formation by autophosphorylation (Schlaepfer et al. 1999). FAK is an important kinase in the mechanotransduction of FSS in hMSCs. Inhibition of FAK blocks osterix transcriptional activity and the osteogenic differentiation of hMSCs. It has also been demonstrated that FAK plays an important role in regulating ECM-induced osteogenic differentiation of hMSCs (Salasznyk et al. 2007b). Pavalko et al. (2003) mentioned a model termed as mechanosomes that comprise ECM, focal adhesion proteins, adherens junctions, cytoskeleton and nuclear matrix. The FSS-induced membrane deformation of cells leads to the conformational change of membrane proteins linking to mechanosomes and, in turn to release protein complexes to conduct mechanical signals.

2.3 Glycocalyx

The cell surface proteoglycan layer, glycocalyx, is considered as a primary sensor for mechanical stimulus. Glycocalyx connects ECM to cellular membrane proteins and lipids by covalent bonds. This connection forms a physical structure to sense the mechanical stimulus loaded on the surface of cells. It has been proved that glycocalyx is necessary for the response of endothelial cells (EC) to FSS and is modulated by the fluid flow (Nijenhuis et al. 2008). FSS can induce the incorporation of hyaluronan in glycocalyx

(Gouverneur et al. 2006). Depletion of heparan sulfate (HS), hyaluronic acid (HA) and sialic acids (SA) blocks shear-induced NO production but has no influence on shear-induced prostaglandin I2 (PGI2) production (Pahakis et al. 2007). Modification of glycocalyx quantity affects significantly both short-term and long-term shear responses (Yao et al. 2007). Glycocalyx can affect the reorganization of actin cytoskeleton. Thi et al. (2004) have pointed out that glycocalyx reorganizes actin cytoskeleton like a bumper-car. The actin cortical web (ACW) and the dense peripheral actin bands (DPABs) are loosely connected to the basal attachment sites. When cells are exposed to FSS, FSS may produce a drag on the tips of core proteins at the edge of glycocalyx and cause a torque on the ACW that consequently leads to a clockwise rotation. All core proteins act in concert to produce a clockwise rotation of DPAB, which creates a disjoining torque that is resisted by vascular cadherins in the adherens junction. When forces and torques exceed the bond strength of vascular cadherins, these bonds rupture, and DPAB gradually breaks up into fragments. At this time, the lateral margins of cells are in an unstable configuration. New focal adherens (FAs) and stress fibers (SFs) need to be formed in the junctional region at the basal margins of cells, which requires the migration of vinculin to the cell borders to form new basal peripheral adhesions. It has also been found that the signals caused by FSS can be transmitted to the basal FAs via SFs that attach at apical plaques, whether glycocalyx is intact or not (Catelas et al. 2006). Therefore, two distinct cellular signaling pathways may be involved in response to FSS: one is transmitted by glycocalyx core proteins as a torque that acts on ACW and DPABs, and the other is emanating from focal adhesions and stress fibers at the basal and apical membranes of the cell. Through these cell surface structures, the signals caused by FSS are conveyed into cells.

Most of evidence for the role of glycocalyx in mechanotransduction comes from the response of EC to FSS (Lopez-Quintero et al. 2009; Nijenhuis et al. 2009; Thi et al. 2004). Lanctot et al. (2007) have found that glycocalyx plays significant role in the differentiation of mesenchymal stem cells. However, it should be remained for further study whether glycocalyx is also an important mechanical sensor for hMSCs to detect FSS like other cells from diverse tissues (Kernan et al. 1994; Sachs 1986).

2.4 Cytoskeleton

FSS can induce the modification of molecular structures in various cells, especially the cytoskeletal proteins (Jaasma et al. 2007). The cytoskeleton is rearranged and the amount of special structural proteins in the cytoskeleton changes in response to FSS (Fu et al. 2008; Jaasma and O'Brien 2008). The rearrangement of the cytoskeleton is critical in the regulation of osteogenic differentiation of hMSCs (Rodriguez et al. 2004). During differentiation of hMSCs, microfilaments change from parallel-oriented actin stress fibers extending across the entire cytoplasm to a cortical organization. Disrupting microfilament with cytochalasin D can decrease the activity of ALP in hMSCs at early stage of osteogenic differentiation. However, no significant change of the microtubule structure takes place between osteoblast and hMSCs (Titushkin and Cho 2007). During the first 5 days of osteogenic differentiation, cells with microtubule destroyed by nocodazole display the same ALP activity as those untreated cells (Rodriguez et al. 2004). Therefore, the main cytoskeleton that plays a critical role in the mechanotransduction during FSS-induced osteogenic differentiation of hMSCs should be actin cytoskeleton (Arnsdorf et al. 2009).

Cytoskeleton plays a key role not only in cell-shape stability but also in signaling pathways that regulate intracellular processes and protein expression in response to a varying biomechanical environment. The role of cytoskeleton as a mechanotransducer has been articulated in the studies of cellular responses to changes of substrate stiffness and cell shape, stretching of cells and shear stress. However, there are significant differences in cytoskeleton reorganization between osteoblasts and hMSCs. HMSCs display many thick actin bundles or stress fibers, extending throughout the cytoplasm and terminating at focal contacts on the cell membrane. In contrast, osteoblasts have fewer stress fibers and show, predominantly, a thin dense meshwork structure of actin. This difference is reflected in the cell elasticity: the Young's modulus for hMSCs is twofold higher than that for osteoblasts (Titushkin and Cho 2007). Cell stiffness is regulated by actin organization. hMSCs cultured on stiff substrate spread out, and cytoskeletal contraction generates high levels of tensile forces that pull on the surface (Patwari and Lee 2008). These changes promote differentiation of stem cells towards the osteoblast lineage. Moreover, the over-expression of either Rho or Rho-associated kinase (Rock), both stimulate contraction of actin cytoskeleton, also promotes osteoblastic differentiation (Arnsdorf et al. 2009). The activation of Rho is mediated by guanine nucleotide exchange factors (GEFs). GEF binds to inactived Rho-GDP to form a Rho-GEF dimer that destabilizes the GDP binding. The upstream signals regulating Rho GTPase activity, such as integrin and TGF-β, may induce the relocalization of GEFs to membrane structures containing the GTPase targets. GTP replaces GDP to activate Rho and GEFs are released from Rho-GTP (Sinha and Yang 2008). Active Rho interacts with Rock and, in turn to phosphorylate myosin phosphatase. Activated myosin phosphatase activates myosin light chain to increase the contraction of actin cytoskeleton (Maekawa et al. 1999). The contraction of actin cytoskeleton is critical for the differentiation of MSCs (Patwari and Lee 2008). Moreover, the activated Rho has an additive effect on Runx2 expression (Arnsdorf et al. 2009). Therefore, the over-expression

of Rho and Rock promotes the osteogeinc differentiation of hMSCs.

3 MMPS and mechanotransduction of FSS in hMSCs

FSS stimulates hMSCs in the form of a biomechanical signal. This biomechanical signal must be translated into the biochemical signal to induce cellular responses. MMPs are thought to play an important role in mechanical signal transformation. MMPs are a family of endoproteinases comprising more than 25 members. Not only can they cleave all components of extracellular matrix but also other substrates such as growth factor binding proteins or latent growth factors, thus regulating their bioavailability (Fig. 1) (Chang and Werb 2001; Kasper et al. 2007; Stamenkovic 2000). The catalytic activities of MMPs depend on metal ions. At least three members of MMPs family, including MMP2, MMP9 and membrane type 1 MMP, affect bone development and homeostasis (Kasper et al. 2007). It was reported that patients with an inherited MMP2 gene mutation exhibited nodulosis, arthropathy and osteolysis syndrome (Martignetti et al. 2001; Parikka et al. 2005; Sternlicht and Werb 2001). Mechanical stimulation can up-regulate activities of MMP2, MMP3, MMP13 and tissue-specific inhibitors of metalloproteases (TIMP2) in hMSC (Charoonpatrapong-Panyayong et al. 2007; Chen et al. 2008; Kang et al. 2008; Kasper et al. 2007). Moreover, the activity of MMP-13 influences osteogenic differentiation of hMSCs and the MMPs/TIMP balance seems to play an important role in transferring mechanical signal into cellular function in hMSCs (Kasper et al. 2007).

MMPs affect hMSC's behavior by degrading ECM component and dissolving mechanical barriers to expose cryptic sites that could act to regulate MSCs' activities (Mott and Werb 2004; Parikka et al. 2005). Furthermore, the modulation of growth factor activity or bioavailability is another likely mechanism of MMPs' impact on MSC behavior. Indeed, MMPs have been shown to modulate important molecules responsible for MSC migration, differentiation, and proliferation, including transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Scutt and Bertram 1999). Therefore, the mechanism by which mechanical stimulation translated into cellular response may be inferred as follows: the balance of MMP/TIMP can be broken by mechanical stimuli (Fehrenbacher et al. 2003; Kasper et al. 2007). The alteration of MMP/TIMP leads to an enhancement of proteolytic enzyme activity, especially gelatinolytic enzyme activity in hMSCs. This may be very important to mechanotransduction in hMSCs. Collagen is the major extracellular matrix component. The enhancement of gelatinolytic enzyme activity results in the breakdown of extracellular matrix components so that the latent growth factors are released. Growth factors can activate intracellular signaling pathways. Accordingly, hMSCs might respond to the stimulation of FSS in an autocrine manner.

4 Intracellular signaling pathways activated by FSS in hMSCs

During osteogenic differentiation of hMSCs induced by FSS, multiple intracellular signaling pathways are activated, such as NO/PKG, PGE2/PKA, Ca^{2+}/PKC and MAPK signaling pathway. Runx2 and AP-1 are important transcription factors in osteogenic differentiation. Their activities can be increased via the aforementioned signaling pathways. Activated Runx2 and AP-1 promote the expression of specific genes, such as alkaline phosphatase (ALP), osteocalcin, collagen I and osteopontin. Consequently, the mineralization of hMSCs is greatly increased (Haasper et al. 2008; Stiehler et al. 2009).

4.1 NO and PGE2

The level of nitric oxide (NO) and prostaglandin E2 (PGE2) in MSCs can be increased in response to FSS (McAllister et al. 2000). The inhibition of nitric oxide synthase (NOS) activity reduces the osteogenic differentiation of MSCs (Knippenberg et al. 2005; Ocarino et al. 2008). In addition, in vivo studies have shown that new bone formation induced by mechanical loading can be blocked by indomethacin, a prostaglandin inhibitor (Pead and Lanyon 1989). Therefore, NO and PGE2 are important to the osteogenic differentiation of MSCs induced by FSS.

NO, a lipophilic molecule, can rapidly diffuse through biological membranes, activate guanylyl cyclase and promote cGMP synthesis (Espanol and Sales 2000). FSS-induced NO response is independent of FSS-induced PGE2 response in osteoblasts and osteocytes. McGarry et al. (2005) found that the fluid flow-induced NO response in osteoblasts was accompanied by parallel alignment of stress fibers, whereas PGE2 response was related to fluid flow stimulation of focal adhesions formed after cytoskeletal disruption. In addition, NO synthesis can lead to activation of cGMP-dependent protein kinase (PKG) by promoting the formation of cGMP. Subsequently, PKG causes the phosphorylation of receptor serine/threonine kinases, increases the activity of ERK, and finally increases the activity of transcription factors, such as Runx2 and AP-1. The activation of these transcription factors promotes the transcription of collagen I, osteocalcin and other special genes involved in osteogenic differentiation (Fig. 1) (Damoulis et al. 2007; Rangaswami et al. 2009). Indeed, some studies showed that NO played an important role in the osteoblastic differentiation of hMSCs (Orciani et al. 2009; Xiao et al. 2001). Collectively, the

NO/cGMP/PKG signal pathway plays a role in FSS-induced osteogenic differentiation of hMSCs.

PGE2 also plays an important role in the osteogenic differentiation of hMSCs induced by mechanical force loading. An early up-regulation of prostaglandin release in response to mechanical stress is associated with the subsequent induction of COX-2. COX-2 mediates conversion of arachidonic acid to PGE2 (Kreke and Goldstein 2004). All PGE2 receptors (EP), including EP1, EP2, EP3 and EP4, are G-protein-coupled receptors (GPCRs). The binding of PGE2 to its receptor leads to the activation of adenyl cyclase, which converts ATP into cAMP and activates cAMP-dependent protein kinase A (PKA) (Fig. 1) (Cherian et al. 2003). PKA elicits an immediate response through the induction of genes such as inhibitor of DNA-binding protein (ID2) and FosB followed by sustained secretion of bone-related cytokines such as bone morphogenetic protein (BMP-2), insulin-like growth factor I (IGF-1) and interleukin 1 (IL-1). These bone-related cytokines can up-regulate the expression of osteogenic differentiation related genes, such as ALP and collagen I (Siddappa et al. 2008). In summary, PGE2 affects the differentiation of hMSCs through cAMP/PKA signal pathway (Kleiveland et al. 2008).

4.2 Ca^{2+} signaling pathway

FSS can increase intracellular Ca^{2+} concentration in hMSCs (Riddle et al. 2006; Stiehler et al. 2009). Ca^{2+} concentration is a key factor in intracellular signaling and regulation of cell functions such as proliferation and differentiation. Cellular responses to Ca^{2+} signals are modulated by Ca^{2+}/calmodulin-dependent protein kinase, such as calmodulin kinase II (CaMKII). The increasing of Ca^{2+} leads to the activation of CaMKII, which subsequently phosphorylates ERK1/2 (Fig. 1) (Shin et al. 2008). Therefore, Ca^{2+} signal can lead to the activation of ERK1/2 signaling pathway, which is critical for osteoblastic differentiation. Phosphorylated ERK1/2 can activate many transcription factors including AP-1 family transcription factors, such as $\Delta FosB$ and FosB. AP-1 family transcription factors are necessary for up-regulation of ostoegenic differentiation-specific genes, such as ALP and osteocalcin (Baba et al. 2003). Additionally, Ca^{2+} can stimulate NOS activity through CaMKII. NOS stimulation may increase the production of NO that is an important signaling molecule for mechanotransduction of FSS in hMSCs (Orciani et al. 2009; Zayzafoon 2006).

4.3 MAPK signaling pathway and mechanotransduction in hMSCs

There is strong evidence that mitogen-activated protein kinase (MAPK) pathways, a complex network of sequential protein kinases, are an important intracellular signaling arm of mechanotransduction (Kim et al. 2007; Rodriguez et al. 2004; Salasznyk et al. 2004). It is believed that all mechanical stimuli result in the activation of MAPK signaling pathway, no matter in osteoblasts, epithelial cells or hMSCs (Alexander et al. 2004; Iqbal and Zaidi 2005; Kim et al. 2007). To date, ERK1/2 and p38 are most widely investigated in FSS-induced mechanotransduction in hMSCs.

ERKI/2 is believed to be essential in FSS-induced mechanotransduction in hMSCs. As shown in Fig. 1, many upstream signal molecules can activate ERK1/2. The high expression of integrins and the activation of FAK promote the activation of Ras through adaptor proteins that contain SH2 and SH3 structures (Salasznyk et al. 2007a,b). Subsequently, the activated Ras initiates MAPK signaling pathway by activating Raf, leading to the activation of MEK1/2 / ERK1/2 pathway. In addition, a high Ca^{2+} concentration, caused by Ca^{2+} influx and intracellular Ca^{2+}release, leads to the elevation of ATP level and PKC activation through CaM. PKC can also phosphorylate ERK1/2 (Iqbal and Zaidi 2005). Moreover, sufficient NO induced by FSS in hMSCs may activate ERK1/2 signaling pathway through NO/cGMP/PKG pathway (Rangaswami et al. 2009). Activated ERK1/2, in turn, activates transcription factors, such as FosB and Runx2, both are necessary for specific gene expression in osteogenic differentiation (Haasper et al. 2008).

FSS can up-regulate the expression of MAP kinase kinase kinase 8 (MAP3K8) and interleukin-1 beta (IL1 β) (Glossop and Cartmell 2009). IL-1 is an activator of the MAPK signaling pathways, acting through the IL-1 receptor and an adapter protein MyD88. My88 recruits a signaling complex, consisting of IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated kinase 6, to the cell membrane (Cao et al. 1996; Glossop and Cartmell 2009; Medzhitov et al. 1998). This leads to the activation of kinase TAK (MAP3K7) to form a complex with TAB proteins and ultimately to activate the JNK and p38 signaling pathway. Rodriguez et al. (2006) reported that IL-1 promotes a 10-fold increase in the induction of MAP3K8. The substrates of MAP3K8 are ERK1/2, JNK and p38. Collectively, the p38 signaling pathway in hMSCs can be activated by FSS. However, the role that the p38 signaling pathway plays in the FSS-induced osteogenic differentiation of hMSCs remains controversial. Simmons et al. (2003) found that p38 seemed to inhibit FSS-induced osteogenic differentiation of hMSCs. The inhibition of p38 pathway resulted in a mature osteogenic phenotype, suggesting an inhibitory role of p38 pathway in modulating FSS-induced osteogenic differentiation of hMSCs. In contrast, Grellier et al. (2009) proposed a different role for p38 in the FSS-induced osteoblastic differentiation of hMSCs. Phosphorylated p38 was markedly increased at 90 min after fluid flow loading when compared with static controls and resulted in a large increase in ALP gene expression. FSS did not induce ALP gene expression in hMSCs treated with

SB203580, a specific inhibitor to p38 MAPK. In conclusion, p38, like ERK1/2, played an active role in FSS-induced osteogenic differentiation of hMSCs.

4.4 Transcription factors related to osteogenic differentiation: Cbfa1/Runx2 and AP-1

Cbfa1/Runx2 and AP-1 are important transcription factors involved in osteogenic differentiation of hMSCs (Bjerre et al. 2008; Haasper et al. 2008; Shin et al. 2008). Runx2 is essential not only in the osteogenic differentiation of hMSCs but also in the differentiation of osteoblasts and chondroblasts (Fu et al. 2007). It is modulated by various signals such as ECM, osteogenic growth factors such as bone morphogenic proteins (BMPs) and fibroblasts growth factor-2 (FGF-2), mechanical loading and hormones (Franceschi and Xiao 2003). In response to FSS, the mRNA and protein levels as well as the DNA-binding ability of Runx2 are augmented *via* ERK1/2/MAKP pathway in hMSCs (Fig. 1) (Kim et al. 2007). Runx2 controls the expression of osteoblast-related genes, such as osteocalcin, ALP, MMP-13, bone sialoprotein and collagen type I α1, by binding to osteoblast-specific cis-acting element (OSE2) in their promoter region of these genes (Ducy et al. 1997).

AP-1 transcription factors are dimers of *Fos (c-Fos, FosB, ΔFosB, Fra-1, Fra-2)* and *Jun (c-Jun, JunB, JunD)* leucine zipper-containing proteins. Some AP-1 factors, such as *c-Fos*, *fosB*, $\Delta FosB$ and *c-jun* can be activated in response to FSS (Haasper et al. 2008; Rangaswami et al. 2009; Young et al. 2009). Similar to Runx2, AP-1 factors can also bind to the consensus sequence in the promoters of several osteogenic differentiation-related genes to regulate their expression (Ducy and Karsenty 1995). As Runx2 can bind to some AP-1 factors, such as *c-Fos, c-Jun* and *JunD* (Swarthout et al. 2002), it is possible that Runx2 and AP-1 factors synergize in regulating the expression of downstream target genes related to osteogenic differentiation though they can also affect these target genes alone.

5 Conclusions

HMSCs are widely used in bone tissue engineering because of their capacity to differentiate into almost all lineages of bone cells. Strategies for bone tissue engineering require collecting hMSCs from the patient and cultivating them in an appropriate environment in vitro to proliferate, differentiate, and generate extracellular matrix prior to reimplantation in bone defects. An ideal artificial bone tissue should have the uniform texture and the capacity to adapt to its functional environment so that its morphology is "optimized" for the mechanical demand (Rubin et al. 2006). FSS produced by proper bioreactors can not only improve the cell seeding distribution, promote proliferation and osteogenic differentiation of hMSCs, but also provide mechanical stimulus to produce bone tissues with a proper resistance against mechanical failure (Chen et al. 2008; Riddle et al. 2006; Zhao et al. 2009). Understanding the molecular mechanism of the osteogenic differentiation of hMSCs induced by FSS will help us to reasonably use FSS in bone tissue engineering. The frequency and strength of FSS should be optimized for the best formation of bone tissue. In addition, findings of FSS study will facilitate us to identify new approaches for treatment of bone diseases, such as osteoporosis and osteoarthritis, and bone injuries. The elucidation of FSS mechanotransduction in hMCSs is also helpful to study the effect of mechanical stress on other cells.

However, the mechanism of FSS-induced osteogenic differentiation in hMSCs is very complex. As shown in Fig. 1, many signal pathways are involved in this mechanotransduction. Yet, the mechanism is not completely clear. There may be other signal pathways related to this mechanism. As undifferentiated osteoprogenitor cells, hMSCs have different characteristics from other cells. It is proposed that the mechanotransduction of hMSCs could be different in some aspects from those of other types of cells, such as osteoblasts and endothelial cells. Further studies should be performed to investigate the mechanism for FSS-induced osteogenic differentiation of hMSCs.

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