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Expression of muscle anabolic and metabolic factors in mechanically loaded MLO-Y4 osteocytes

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Juffer P, Jaspers RT, Lips P, Bakker AD, Klein-Nulend J. Expression of muscle anabolic and metabolic factors in mechanically loaded MLO-Y4 osteocytes. *Am J Physiol Endocrinol Metab* 302: E389–E395, 2012. First published November 22, 2011; doi:10.1152/ajpendo.00320.2011.—Lack of physical activity results in muscle atrophy and bone loss, which can be counteracted by mechanical loading. Similar molecular signaling pathways are involved in the adaptation of muscle and bone mass to mechanical loading. Whether anabolic and metabolic factors regulating muscle mass, i.e., insulin-like growth factor-I isoforms (IGF-I Ea), mechano growth factor (MGF), myostatin, vascular endothelial growth factor (VEGF), or hepatocyte growth factor (HGF), are also produced by osteocytes in bone in response to mechanical loading is largely unknown. Therefore, we investigated whether mechanical loading by pulsating fluid flow (PFF) modulates the mRNA and/or protein levels of muscle anabolic and metabolic factors in MLO-Y4 osteocytes. Unloaded MLO-Y4 osteocytes expressed mRNA of VEGF, HGF, IGF-I Ea, and MGF, but not myostatin. PFF increased mRNA levels of IGF-I Ea (2.1-fold) and MGF (2.0-fold) at a peak shear stress rate of 44Pa/s, but not at 22Pa/s. PFF at 22 Pa/s increased VEGF mRNA levels (1.8- to 2.5-fold) and VEGF protein release (2.0- to 2.9-fold). Inhibition of nitric oxide production decreased (2.0-fold) PFF-induced VEGF protein release. PFF at 22 Pa/s decreased HGF mRNA levels (1.5-fold) but increased HGF protein release (2.3-fold). PFF-induced HGF protein release was nitric oxide dependent. Our data show that mechanically loaded MLO-Y4 osteocytes differentially express anabolic and metabolic factors involved in the adaptive response of muscle to mechanical loading (i.e., IGF-I Ea, MGF, VEGF, and HGF). Similarly to muscle fibers, mechanical loading enhanced expression levels of these growth factors in MLO-Y4 osteocytes. Although in MLO-Y4 osteocytes expression levels of IGF-I Ea and MGF of myostatin were very low or absent, it is known that the activity of osteoblasts and osteoclasts is strongly affected by them. The abundant expression levels of these factors in muscle cells, in combination with low expression in MLO-Y4 osteocytes, provide a possibility that growth factors expressed in muscle could affect signaling in bone cells.

osteocytes; mechanical loading; bone remodeling; signaling pathways

PHYSICAL INACTIVITY, AS OCCURS DURING AGING, chronic disease, or skeletal injury, is associated with osteoporosis and sarcopenia (45, 48). Loss of bone mass and muscle mass can be reversed by increasing the rate at which bone cells and muscle cells synthesize proteins and/or attenuate the rate of protein degradation (58). Mechanical loading of muscle and bone results in cellular adaptation via mechanotransduction. This alters the rate of protein turnover by activation of signaling pathways regulating the rate of protein synthesis and/or the rate of protein degradation (58). Alternatively, mechanical loading may cause changes in the expression of anabolic and metabolic growth factors that affect muscle and/or bone cells via autocrine, paracrine, and/or endocrine systems (2, 25).

Skeletal muscle mass and oxidative capacity are determined by the net difference in the rates of synthesis and degradation of contractile and mitochondrial proteins within a muscle fiber. Muscle size and oxidative capacity are inversely related (58). This implicates that muscle size is regulated such that supply and demand of nutrients and oxygen are matching to meet functional demand and prevent hypoxia. Since muscle fibers are multinucleated, the rate of protein synthesis may vary with the number of myonuclei (46). Changes in muscle contractile activity stimulate expression of factors that modulate muscle mass by activating satellite cell proliferation by affecting the rate of mRNA transcription, mRNA translation, or protein degradation (6). Hence, a loss or gain of skeletal muscle mass and/or oxidative capacity follows.

Numerous growth factors are capable of modulating the adaptation of muscle fiber size and oxidative capacity. The prime anabolic and metabolic growth factors involved in muscle hypertrophy include insulin-like growth factor-I (IGF-I) isoforms IGF-I Ea and mechano growth factor (MGF) (22), hepatocyte growth factor (HGF) (51), vascular endothelial growth factor (VEGF) (16), and myostatin (41). Expression of these growth factors is determined largely by mechanical loading of muscle fibers (22).

In contrast to muscle, in which the systems for synthesis and degradation of the contractile apparatus all reside within the muscle cell, bone remodeling requires three different cell types, i.e., osteoblasts, osteoclasts, and osteocytes (57). During bone remodeling, bone is constantly renewed by the activity of osteoclasts, which remove excess bone in places that are relatively unloaded, and osteoblasts, which form bone in places that are exposed to loading (37, 57). The initiation of mechanically induced bone formation and bone resorption is orchestrated by the osteocytes and the mechanosensing cells of bone (11, 57), although cells from other tissues associated with the musculoskeletal system can affect bone formation, e.g., bone morphogenic protein 4 production by tenocytes in tendons (7).

Similarly to muscle fibers, which produce growth factors to adapt muscle mass to mechanical loading, osteocytes produce growth factors to orchestrate the adaptation of bone mass and structure to mechanical loading (55, 57, 59). Since muscle fibers and osteocytes share the same mesenchymal precursor

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cell, and since mechanical loading affects both muscle and bone by increasing tissue mass and strength, it is plausible that both muscle fibers and osteocytes share similar biological pathways in the regulation of muscle fiber size and bone mass and structure in response to mechanical loading (25, 33).

Although IGF-I Ea, MGF, VEGF, HGF, and myostatin have been reported to affect the effector cells involved in bone remodeling (2, 9, 14, 20, 24, 26, 49, 56, 60), it is largely unknown whether osteocytes produce these factors in response to mechanical loading. Therefore, we aimed to determine whether MLO-Y4 osteocytes express basal levels of the most widely studied muscle anabolic and metabolic factors and to assess whether mechanical loading by pulsating fluid flow (PFF) affects the mRNA levels and/or protein levels of these growth factors in MLO-Y4 osteocytes.

MATERIALS AND METHODS

Osteocyte culture. MLO-Y4 osteocytes were cultured in α-minimal essential medium (α-MEM; Gibco, Paisly, UK) supplemented with 10 μg/ml penicillin (Sigma-Aldrich, St. Louis, MO), 10 μg/ml streptomycin (Sigma-Aldrich), 50 μg/ml fungizone (Gibco), 5% fetal bovine serum (FBS; Gibco), and 5% calf serum (CS; Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was exchanged every 3–4 days. The MLO-Y4 osteocytes were kindly provided by Dr. L. F. Bonewald (University of Missouri-Kansas City, Kansas City, MO) (34). Upon confluence, cells were harvested using 0.25% trypsin and 0.1% EDTA in phosphate-buffered saline seeded at 2×10^5 cells/75 cm^2 culture flask (Greiner Bio-One, Kremsmuenster, Austria) and cultured until the cell layer reached confluence again. MLO-Y4 osteocytes were used for PFF experiments, as described below.

PFF. One day before PFF treatment, MLO-Y4 osteocytes were harvested from the culture flasks and seeded onto polylysine-coated (50 μg/ml poly-L-lysine hydrobromide; Sigma) glass slides (size 2.5 × 6.5 cm) at 3×10^5 cells/glass slide and cultured overnight in petridishes with 13 ml of culture medium containing 5% FBS and 5% CS. Then, the culture medium was replaced by α-MEM medium containing 1% FBS and 1% CS. MLO-Y4 osteocytes were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air as well as for 1 h PFF treatment or static control culture.

PFF was applied as described earlier (36). Briefly, PFF was generated by pumping 13 ml of culture medium through a parallel-plate flow chamber containing the MLO-Y4 osteocytes. The cells were subjected to a 5-Hz pulse frequency with a mean shear stress of 0.7 Pa, a pulse amplitude of 0.3 Pa, and a peak shear stress rate of 22 Pa/s (5). Higher shear stresses were generated using a 5-Hz pulse frequency with a mean shear stress of 1.4 Pa, a pulse amplitude of 1.4 Pa, and a peak shear stress rate of 44 Pa/s. These loading regimes were carefully designed to mimic the fluid shear stress magnitude (ranging from 0.8 to 3.0 Pa) that osteocytes experience in vivo (62). Static control cultures were kept in a petri dish under conditions similar to the experimental cultures, i.e., at 37°C in a humidified atmosphere of 5% CO_2 in air. After 1 h of PFF treatment or static control culture, MLO-Y4 cells were postincubated for 1, 3, 6, and 24 h.

Inhibition of nitric oxide synthesis. Nitric oxide (NO) production was inhibited by adding 10 μM 1400W to the culture medium 1 h prior to PFF treatment, during PFF treatment, and during unloaded postincubation.

Inhibition of protein synthesis. One of the major signaling molecules in protein synthesis is mammalian target of rapamycin (mTOR). mTOR-mediated protein synthesis was inhibited by adding rapamycin (0, 5, and 50 μM) to the culture medium during PFF treatment and postincubations.

NO production. NO production was measured as nitrite (NO_2^-) accumulation in the conditioned medium using Griess reagent containing 1% sulfanylamide, 0.1% naphtylethelene-diamine-dihydrochloride, and 2.5 M H_3PO_4. Serial dilutions of $NaNO_2$ in nonconditioned medium were used as a standard curve. Absorbance was measured at 540 nm with a microplate reader (Bio-Rad Laboratories).

RNA isolation and real-time PCR. Total RNA was isolated using the RiboPure Kit (Applied Biosystems, Foster City, CA). Total RNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, DE). mRNA was reverse-transcribed to complementary DNA (cDNA) using a High Capacity RNA-to-cDNA Kit (Applied Biosystems). Real-time PCR for determination of mouse IGF-I Ea, MGF, VEGF, HGF, and myostatin mRNA was performed on the StepOne Real-Time PCR system (Applied Biosystems). Primers were designed using the Universal Probe Library from Roche Diagnostics. Primers were as follows: IGF-I Ea, 5′-GTGTTGCTTCCGGAGCTGTG and 5′-CAAATGTACTTCCTTCTGAGTC; MGF, 5′-GGAGAAGGAAAGGAAGTACATTTG and 5′-CCTGCTCCGTGGGAGGCT; VEGF, 5′-CTGTAACGATGAAGCCCTGGAGTG and 5′-GGTGAGGTTTGATCCGCATGATCT; HGF, 5′-GATTATTGCCCTATTTCCCGTTGTG and 5′-TGGCACAGGATATTACAGGATGG; myostatin, 5′-TGCTGTAACCTTCCCAGGACCA and 5′-GTGAGGGGGTAGCGGCAGCAC; and 18S, 5′-GTAACCCGTTGAACCCCATT and 5′-CCATCCAATCGGTAGTAGCG. Data were analyzed using StepOne version 2.0 software (Applied Biosystems) and normalized for 18S ribosomal RNA levels.

Protein release. VEGF, HGF, and IGF-I protein levels in conditioned medium were measured by an enzyme-linked immunosorbent assay (RayBio Mouse ELISA kit; RayBiotech). The detection limits were as follows: VEGF, 2 pg/ml; HGF, 100 pg/ml; and IGF-I, 4 pg/ml. Absorbance was measured at 450 nm with a microplate reader (Bio-Rad Laboratories).

Statistical analysis. Data of five separate independent experiments were obtained. Statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL). Groups were compared using ANOVAs with Bonferroni-adjusted *t*-tests as post hoc tests. Differences were considered significant if $P < 0.05$. All data are expressed as means ± SE.

RESULTS AND DISCUSSION

Expression of muscle anabolic and metabolic factors by MLO-Y4 osteocytes. Force transmission from muscle via its tendons to bone is presumed to be one of the primary mechanisms by which muscle mass affects bone remodeling (50). However, muscle may also modulate the rate of bone turnover via expression and secretion of growth factors that stimulate osteogenesis via paracrine or endocrine signaling (2, 25) Here, we show that anabolic and metabolic regulatory growth factors in muscle fiber size, i.e., IGF-I Ea, MGF, VEGF, and HGF, are also basally expressed in MLO-Y4 osteocytes (Fig. 1, *A* and *B*). Expression levels of VEGF and HGF mRNA relative to 18S in MLO-Y4 osteocytes are in the same order of magnitude as those reported for muscle cells (28, 39). In contrast, expression levels of IGF-I Ea and MGF mRNA are substantially lower (i.e., 10,000-fold) compared with those in muscle cells (31). In addition, basal mRNA levels of the negative regulator of muscle growth myostatin were not detectable in MLO-Y4 osteocytes (Fig. 1*A*), although myostatin is abundantly expressed in muscle cells (27). This suggests that the reported inhibitory effects of myostatin on proliferation and differentiation of osteoprogenitor cells (18) likely originate from myostatin expression in muscle. This supports the notion that myostatin is a muscle-specific transforming growth factor-β and hence, supports the presumed existence of paracrine and/or endocrine communication between muscle and bone (2, 25, 33).

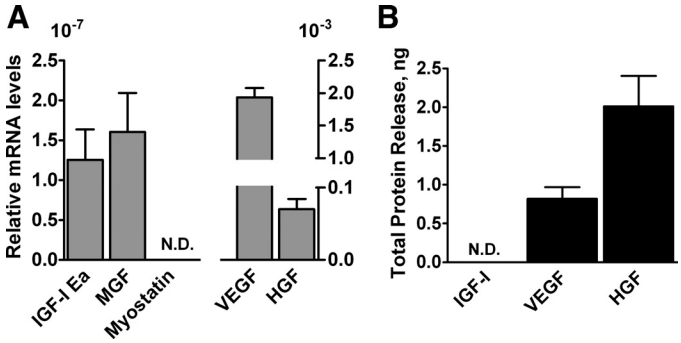

Fig. 1. Expression of muscle anabolic and metabolic factors by MLO-Y4 osteocytes. *A*: MLO-Y4 osteocytes express IGF-I isoform (IGF-I Ea), mechano growth factor (MGF), VEGF, and hepatocyte growth factor (HGF) mRNA. Myostatin mRNA levels were not detectable (ND). *B*: total protein release during 24-h culture. MLO-Y4 osteocytes secrete VEGF and HGF protein. IGF-I protein was ND. Values are means ± SE of untreated MLO-Y4 osteocytes. mRNA levels were normalized for 18S ribosomal RNA levels.

Mechanosensitivity and bone remodeling: role of muscle anabolic and metabolic factors. Osteocytes play a central role in bone remodeling by sensing the external mechanical stimuli and transducing these into signaling molecules like NO and prostaglandins (35, 36), which modulate the activity of osteoblasts and/or osteoclasts. It has already been reported that anabolic and metabolic growth factors, which play a role in the regulation of muscle fiber size, i.e., IGF-I Ea, MGF, myostatin, VEGF, and HGF, also affect the activity of osteoblasts and/or osteoclasts (2, 9, 14, 15, 20, 24, 26, 49, 56, 60). Since osteocytes coordinate the activity of osteoblasts and osteoclasts in the process of mechanical adaptation of bone (57), and muscle anabolic and metabolic factors are also expressed in osteocytes, these factors could also play a role in mechanically induced bone remodeling.

In this study, MLO-Y4 osteocytes were subjected to PFF. Application of PFF for 1 h to MLO-Y4 osteocytes did not result in visible changes in cell shape or alignment of the cells in a particular orientation (data not shown). No cells were removed by the fluid flow treatment, as assessed by visually inspecting the cultures before and after PFF treatment.

IGF-I Ea and MGF mRNA levels. In the current study, mechanical loading of MLO-Y4 osteocytes by PFF with a shear stress rate of 22 Pa/s did not increase mRNA levels of IGF-I Ea or MGF (Fig. 2, *A* and *C*). Moreover, IGF-I protein production could not be quantified before or after PFF treatment (data not shown). Loading of rat tibia in situ increases IGF-I mRNA levels in young osteocytes situated near the periosteal surface, where IGF-I mRNA is also highly expressed by the osteoblasts (47). Additionally, IGF-I levels are upregulated in mechanically loaded tibia of ovariectomized mice (67), when RNA is isolated from whole tibiae. The discrepancy between the effects of mechanical loading on IGF-I gene levels in tibia in vivo and our results could be explained by the cell model we used, the type and/or magnitude of load, and/or the differences between in vitro and in vivo loading. Osteoblasts have been shown to express MGF in response to stretch (56). However, to our knowledge, MGF mRNA levels in mechanically loaded osteocytes have not been studied before. Since strain and fluid shear stress may activate different mechanosensitive signaling pathways in the cell (42), it is conceivable that not fluid shear stress but strain affects IGF-I Ea and/or MGF mRNA levels by osteocytes. Alternatively, like in muscle (30), local strain amplifications in the cytoskeleton may also occur in bone (66). Therefore, osteocytes in a 3D matrix in

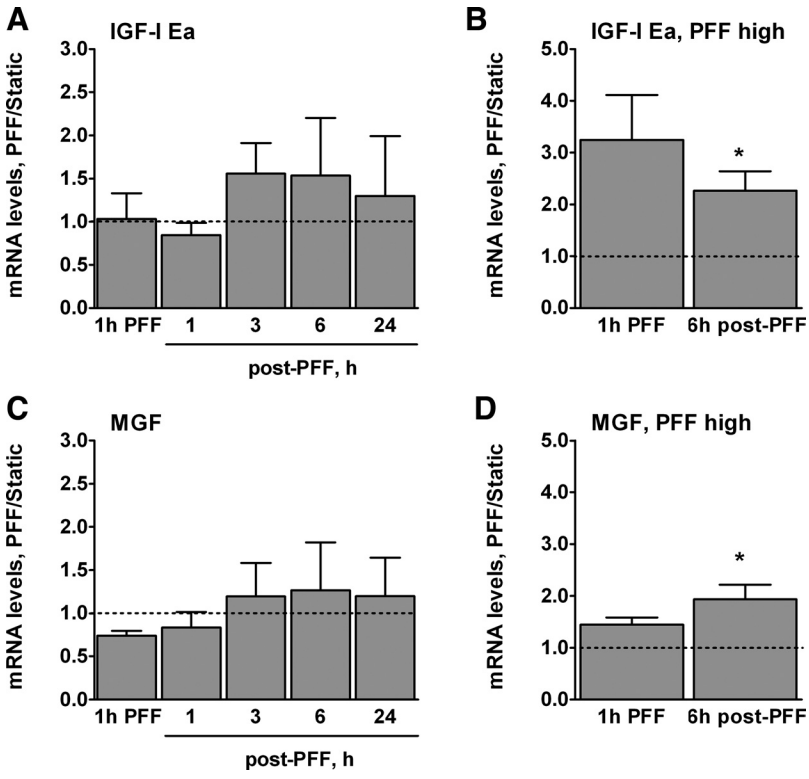

Fig. 2. Effect of pulsating fluid flow (PFF) on IGF-I Ea and MGF mRNA levels in MLO-Y4 osteocytes. Cells were subjected to PFF for 1 h (1-h PFF) and postincubated (without PFF) for 1, 3, 6, and 24 h. PFF at a peak shear stress rate of 22 Pa/s does not significantly change IGF-I Ea mRNA (*A*) or MGF mRNA levels (*C*) in MLO-Y4 osteocytes. Increasing the peak shear stress rate to 44 Pa/s increased both IGF-I Ea (*B*) and MGF (*D*) mRNA levels. Values are means ± SE of PFF-treated/over-control ratios (PFF, $n = 5$; PFF high, $n = 4$). Dashed line, no effect of PFF. Significant effect of PFF; $*P < 0.05$.

vivo may be subjected to higher-intensity mechanical stimuli than osteocytes in a 2D environment. To test whether a higher magnitude of shear stress affects mRNA levels of IGF-I Ea and/or MGF in osteocytes, we subjected MLO-Y4 osteocytes to PFF with a twofold higher peak shear stress rate (i.e., 44 Pa/s). Indeed, higher loads did increase mRNA levels of both IGF-I Ea (2.1-fold) and MGF (2.0-fold) in MLO-Y4 osteocytes (Fig. 2, *B* and *D*). This suggests that, in vivo, increased mRNA levels of IGF-I Ea and MGF in osteocytes are to be expected when higher shear stresses are experienced by the osteocytes.

In muscle, IGF-I Ea is a potent growth factor that stimulates both the rate of mRNA transcription of contractile proteins (53) and their rate of translation via phosphatidylinositol-3 kinase/Akt/mTOR pathway (32). The splice variant of IGF-I, MGF, seems to be involved in satellite cell activation and migration of myoblasts and satellite cells, resulting in an increased myonuclear density in the muscle fiber (44, 65). In muscle, mRNA levels of both isoforms, IGF-I Ea and MGF, are upregulated by mechanical loading (27). The anabolic effects of IGF-I Ea and MGF on bone are less well characterized (19). In bone, MGF E-peptide (a 23-amino acid peptide encoded the MGF E-domain) stimulates osteoblast proliferation, whereas IGF-I Ea stimulates both osteoblast proliferation and differentiation (15, 40). Additionally, recombinant IGF-I has been shown to stimulate osteoclast differentiation and resorption activity (25, 63), indicating that IGF-I Ea may increase not only bone formation but also bone resorption.

Bone formation and bone resorption: role of HGF. We found that mechanical loading by PFF decreased HGF mRNA levels in MLO-Y4 osteocytes (Fig. 3*A*). This decrease did not result in a decrease in HGF protein release, since PFF even increased HGF protein release 2.3-fold (Fig. 3*B*). These results suggest that the PFF-increased HGF protein levels in the medium were due to enhancement of translation of HGF mRNA or protein release from the glycocalyx. To test whether the increase of HGF protein levels in the medium was caused by an increased rate of translation, we inhibited phosphorylation of mTOR by adding rapamycin to the culture medium. Rapamycin is a well-known inhibitor of mTOR, thereby inhibiting mTOR-mediated protein translation (8). In the presence of rapamycin, the PFF-induced HGF protein production/release was not abolished (Fig. 3*C*), which indicates that PFF did not increase HGF protein release via mTOR-stimulated translational control. An alternative explanation for the discrepancy between changes in HGF mRNA levels and HGF protein levels in MLO-Y4 osteocytes after PFF-treatment may be that, similarly to muscle fibers, HGF stored in the glycocalyx is released from it by the mechanical loading-induced NO production (4). Such release seems possible for osteocytes, since both muscle fibers and osteocytes produce NO in response to mechanical

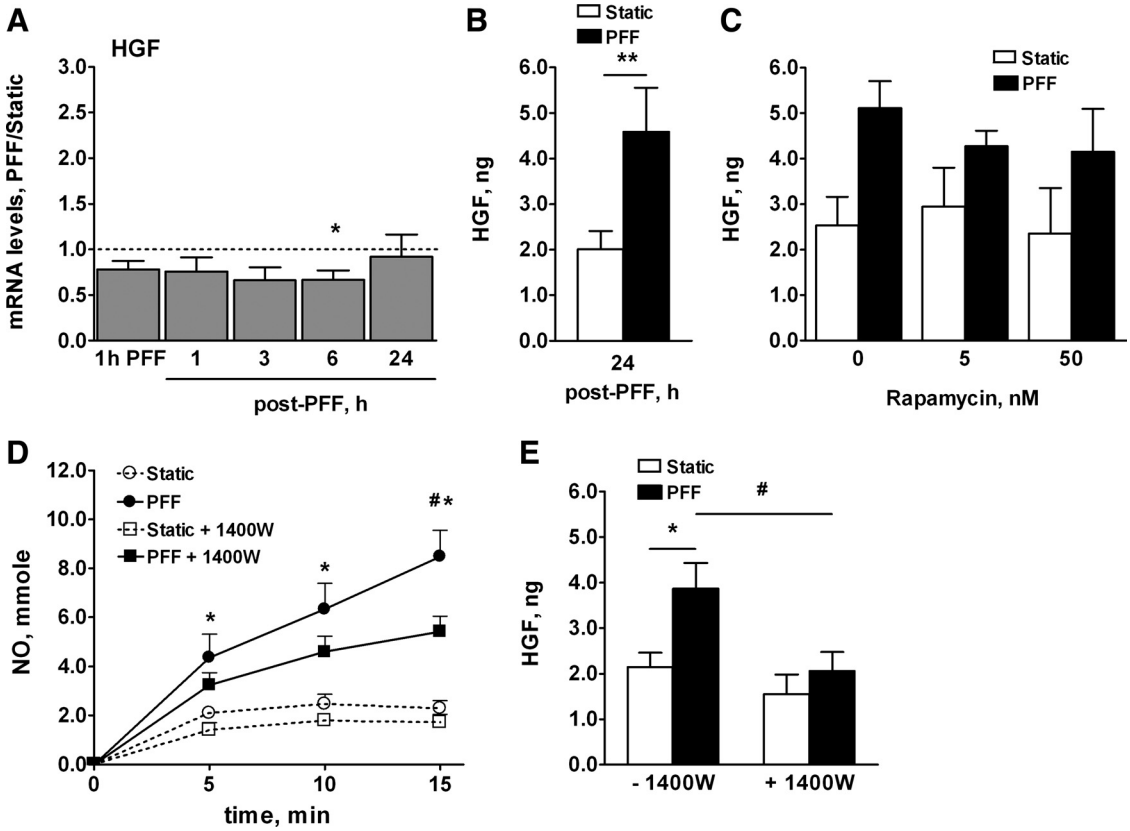

Fig. 3. PFF decreases HGF mRNA levels but increases HGF protein release in MLO-Y4 osteocytes. Cells were subjected to 1-h PFF and postincubated (without PFF) for 1, 3, 6, and 24 h. *A*: HGF mRNA levels. *B*: total HGF protein release per culture. *C*: addition of rapamycin, an inhibitor of protein synthesis, did not affect PFF-induced HGF protein release. *D*: 1400W inhibits the PFF-induced nitric oxide (NO) synthesis at 15 min after PFF treatment. *E*: addition of NO synthase inhibitor 1400W decreased PFF-induced HGF protein release at 24 h postincubation. Values are means ± SE of PFF-treated/over-control ratios or total protein [$n = 5$ (*A* and *B*), $n = 3$ (*C*), and $n = 4$ (*D* and *E*)]. Dashed line, no effect of PFF. Significant effect of PFF; *$P < 0.05$. Significant effect of 1400W; #$P < 0.05$.

loading (13, 63). Therefore, we tested whether the PFF-induced HGF protein release by MLO-Y4 osteocytes was mediated by NO. For this purpose, PFF-induced NO production was inhibited by adding the NO synthase inhibitor 1400W (Fig. 3*D*). Inhibition of NO synthesis decreased the PFF-induced HGF protein release 2.0-fold (Fig. 3*E*), suggesting that NO modulates HGF protein secretion by osteocytes in response to mechanical loading.

HGF plays a role in bone formation, because it stimulates osteoblast differentiation (29) and inhibits mineralization (54). HGF is also involved in bone resorption, because it stimulates osteoclast formation (1, 23). Osteoclastic bone resorption is generally followed by osteoblast differentiation and new bone deposition, i.e., the activity of osteoclasts and osteoblasts is tightly coupled (3). Since it is likely that osteocytes orchestrate the activity of osteoclasts and osteoblasts in the process of mechanical adaptation of bone (10, 55, 57, 59), bone remodeling might be mediated by HGF produced by mechanically loaded osteocytes. The impact of HGF on bone remodeling and the precise role of HGF in this warrant further investigation.

Angiogenesis in bone: role of VEGF. In our study, we show that VEGF mRNA levels and VEGF protein release in MLO-Y4 osteocytes were increased in response to PFF (Fig. 4, *A* and *B*). VEGF expression is also upregulated in mechanicallliy loaded tibia of ovariectomized mice (67). Since VEGF mRNA levels in muscle and endothelial cells are increased by NO (21), we tested whether the PFF-induced VEGF protein release by MLO-Y4 osteocytes was also mediated by NO. Inhibition of NO synthesis by 1400W decreased the PFF-induced VEGF protein release twofold (Fig. 4*C*), suggesting that VEGF expression by osteocytes in response to mechanical loading is partially modulated by NO like in muscle.

Vascularization in muscle is very important for the supply of nutrients and oxygen to muscle fibers (17). A limited supply implicates a low fatigue resistance, which becomes apparent via a reduction in the ability to generate force (17). Similarly, as in muscle, a high demand of nutrients and oxygen is to be expected in bone, particularly since in vitro hypoxia activates osteoblasts to form bone (61). During bone remodeling, resorption and formation of the bone matrix are generally accompanied by angiogenesis in the cutting cone of the osteon (3). It has been reported recently that VEGF, a potent regulator of angiogenesis, is produced by osteocytes (12). Since bone remodeling is induced mechanically, and since we showed that mechanical loading stimulates VEGF mRNA levels and protein release in MLO-Y4 osteocytes, this might implicate that the mechanically induced VEGF production contributes to the vascularization within the cutting cone of the osteon.

VEGF not only stimulates blood vessel formation but also increases bone formation by stimulating osteoblastogenesis (20). Therefore, the PFF-induced VEGF production in osteocytes is likely not only contributing to the vascularization within the bone matrix but is also involved in mechanically induced bone remodeling.

Summary. The present data show that mechanically loaded MLO-Y4 osteocytes differentially express mRNA and protein of anabolic and metabolic factors involved in the adaptive response of muscle to mechanical loading, i.e., IGF-I Ea, MGF, VEGF, and HGF. Comparison of these results with those in muscle suggest similarities as well as differences in signaling pathways in bone and muscle in response to mechanical loading. Mechanical loading affects VEGF and HGF mRNA and protein levels in osteocytes, which is also known for muscle fibers. This indicates novel roles for VEGF and HGF in mechanical loading-mediated bone remodeling. We also show that mechanical loading by PFF increases IGF-I Ea and MGF mRNA levels in MLO-Y4 osteocytes at a high physiological shear stress rate, but these mRNA levels are still several orders of magnitude lower than IGF-I Ea and MGF mRNA levels in muscle (31). MLO-Y4 osteocytes, either subjected to PFF or not, do not express myostatin mRNA. In muscle, IGF-I Ea, MGF, and myostatin are highly expressed (31, 38), and the effect of mechanical loading on the expression of these growth factors is substantial (26). Although in MLO-Y4 osteocytes expression levels of IGF-I Ea and MGF were shown to be low and that of myostatin was not detectable, the activity of osteoblasts and osteoclasts is well known to be strongly affected by these growth factors (14, 15, 23–25, 43, 52, 60, 64). This, in combination with the abundant expression levels of these factors in muscle cells, suggests that osteoblasts and osteoclasts may, in addition to the growth factors expressed in these cells, also respond to muscle-derived IGF-I and myosta-

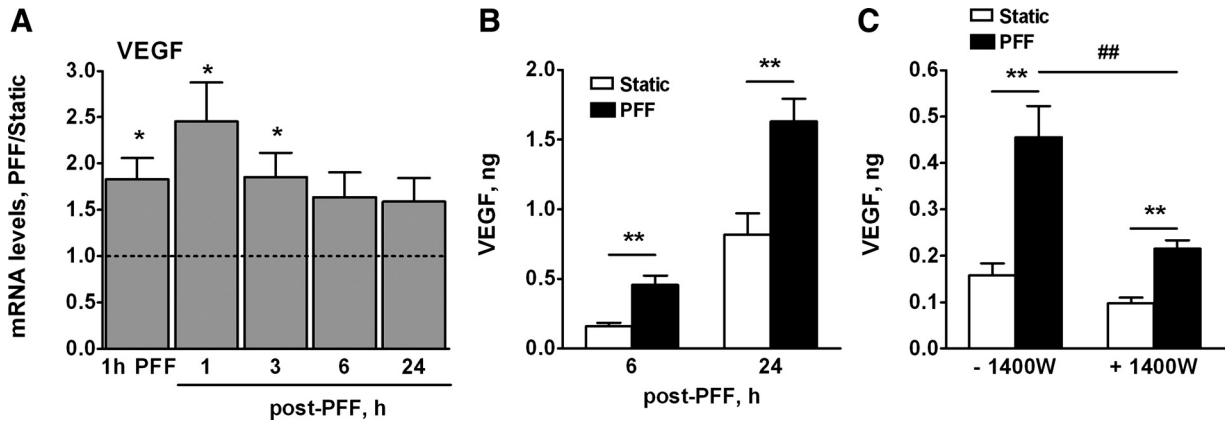

Fig. 4. PFF increases VEGF mRNA levels and VEGF protein release in MLO-Y4 osteocytes. Cells were subjected to PFF for 1 h (1-h PFF) and postincubated (without PFF) for 1, 3, 6, and 24 h. *A*: VEGF mRNA levels. *B*: total VEGF protein release per culture. *C*: addition of NO synthase inhibitor 1400W decreased PFF-induced VEGF protein release at 6 h postincubation. Values are means ± SE of PFF-treated/over-control ratios or total protein ($n = 5$). Dashed line, no effect of PFF. Significant effect of PFF; *$P < 0.05$ and **$P < 0.01$. Significant effect of 1400W; ##$P < 0.01$.

tin. Our results do not provide direct evidence for any paracrine and/or endocrine signaling between muscle and bone, but such communication may not be excluded. Whether muscle-derived growth factors affect bone cells and vice versa requires further investigation. Additional in vivo research on the biological cross-talk between bone cells and muscle cells may reveal new targets for prevention and/or treatment of sarcopenia and osteoporosis.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

1. **Adamopoulos IE, Xia Z, Lau YS, Athanasou NA.** Hepatocyte growth factor can substitute for M-CSF to support osteoclastogenesis. *Biochem Biophys Res Commun* 350: 478–483, 2006.
2. **Alzghoul MB, Gerrard D, Watkins BA, Hannon K.** Ectopic expression of IGF-I and Shh by skeletal muscle inhibits disuse-mediated skeletal muscle atrophy and bone osteopenia in vivo. *FASEB J* 18: 221–223, 2004.
3. **Andersen TL, Sondergaard TE, Skorzynska KE, Dagnaes-Hansen F, Plesner TL, Hauge EM, Plesner T, Delaisse JM.** A physical mechanism for coupling bone resorption and formation in adult human bone. *Am J Pathol* 174: 239–247, 2009.
4. **Anderson J, Pilipowicz O.** Activation of muscle satellite cells in single-fiber cultures. *Nitric Oxide* 7: 36–41, 2002.
5. **Bacabac RG, Smit TH, Cowin SC, Van Loon JJ, Nieuwstadt FT, Heethaar R, Klein-Nulend J.** Dynamic shear stress in parallel-plate flow chambers. *J Biomech* 38: 159–167, 2005.
6. **Bamman MM, Petrella JK, Kim JS, Mayhew DL, Cross JM.** Cluster analysis tests the importance of myogenic gene expression during myofiber hypertrophy in humans. *J Appl Physiol* 102: 2232–2239, 2007.
7. **Blitz E, Viukov S, Sharir A, Shwartz Y, Galloway JL, Pryce BA, Johnson RL, Tabin CJ, Schweitzer R, Zelzer E.** Bone ridge patterning during musculoskeletal assembly is mediated through SCX regulation of Bmp4 at the tendon-skeleton junction. *Dev Cell* 17: 861–873, 2009.
8. **Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD.** Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat Cell Biol* 3: 1014–1019, 2001.
9. **Boudignon BM, Bikle DD, Kurimoto P, Elalieh H, Nishida S, Wang Y, Burghardt A, Majumdar S, Orwoll BE, Rosen C, Halloran BP.** Insulin-like growth factor I stimulates recovery of bone lost after a period of skeletal unloading. *J Appl Physiol* 103: 125–131, 2007.
10. **Burger EH, Klein-Nulend J, Smit TH.** Strain-derived canalicular fluid flow regulates osteoclast activity in a remodelling osteon—a proposal. *J Biomech* 36: 1453–1459, 2003.
11. **Burger EH, Klein-Nulend J, van der Plas A, Nijweide PJ.** Function of osteocytes in bone—their role in mechanotransduction. *J Nutr* 125: 2020S–2023S, 1995.
12. **Cheung WY, Liu C, Tonelli-Zasarsky RM, Simmons CA, You L.** Osteocyte apoptosis is mechanically regulated and induces angiogenesis in vitro. *J Orthop Res* 29: 523–530, 2011.
13. **Cowin SC, Moss-Salentijn L, Moss ML.** Candidates for the mechanosensory system in bone. *J Biomech Eng* 113: 191–197, 1991.
14. **Deckers MM, Karperien M, van der Bent C, Yamashita T, Papapoulos SE, Lowik CW.** Expression of vascular endothelial growth factors and their receptors during osteoblast differentiation. *Endocrinology* 141: 1667–1674, 2000.
15. **Deng M, Zhang B, Wang K, Liu F, Xiao H, Zhao J, Liu P, Li Y, Lin F, Wang Y.** Mechano growth factor E peptide promotes osteoblasts proliferation and bone-defect healing in rabbits. *Int Orthop* 35: 1099–1106, 2011.
16. **Egginton S, Badr I, Williams J, Hauton D, Baan GC, Jaspers RT.** Physiological angiogenesis is a graded, not threshold, response. *J Physiol* 589: 195–206, 2011.
17. **Egginton S, Hudlicka O, Brown MD, Walter H, Weiss JB, Bate A.** Capillary growth in relation to blood flow and performance in overloaded rat skeletal muscle. *J Appl Physiol* 85: 2025–2032, 1998.
18. **Elkasrawy MN, Hamrick MW.** Myostatin (GDF-8) as a key factor linking muscle mass and bone structure. *J Musculoskelet Neuronal Interact* 10: 56–63, 2010.
19. **Fowlkes JL, Thrailkill KM, Liu L, Wahl EC, Bunn RC, Cockrell GE, Perrien DS, Aronson J, Lumpkin CK Jr.** Effects of systemic and local administration of recombinant human IGF-I (rhIGF-I) on de novo bone formation in an aged mouse model. *J Bone Miner Res* 21: 1359–1366, 2006.
20. **Furumatsu T, Shen ZN, Kawai A, Nishida K, Manabe H, Oohashi T, Inoue H, Ninomiya Y.** Vascular endothelial growth factor principally acts as the main angiogenic factor in the early stage of human osteoblastogenesis. *J Biochem* 133: 633–639, 2003.
21. **Gavin TP, Spector DA, Wagner H, Breen EC, Wagner PD.** Nitric oxide synthase inhibition attenuates the skeletal muscle VEGF mRNA response to exercise. *J Appl Physiol* 88: 1192–1198, 2000.
22. **Goldspink G.** Mechanical signals, IGF-I gene splicing, and muscle adaptation. *Physiology (Bethesda)* 20: 232–238, 2005.
23. **Grano M, Gelimi F, Zambonin G, Colucci S, Cottone E, Zambonin Zallone A, Comoglio P.** Hepatocyte growth factor is a coupling factor for osteoclasts and osteoblasts in vitro. *Proc Natl Acad Sci USA* 93: 7644–7648, 1996.
24. **Guicheux J, Heymann D, Rousselle AV, Gouin F, Pilet P, Yamada S, Daculsi G.** Growth hormone stimulatory effects on osteoclastic resorption are partly mediated by insulin-like growth factor I: an in vitro study. *Bone* 22: 25–31, 1998.
25. **Hamrick MW, McNeil PL, Patterson SL.** Role of muscle-derived growth factors in bone formation. *J Musculoskelet Neuronal Interact* 10: 64–70, 2010.
26. **Hamrick MW, Shi X, Zhang W, Pennington C, Thakore H, Haque M, Kang B, Isales CM, Fulzele S, Wenger KH.** Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone* 40: 1544–1553, 2007.
27. **Heinemeier KM, Olesen JL, Schjerling P, Haddad F, Langberg H, Baldwin KM, Kjaer M.** Short-term strength training and the expression of myostatin and IGF-I isoforms in rat muscle and tendon: differential effects of specific contraction types. *J Appl Physiol* 102: 573–581, 2007.
28. **Honda H, Abe S, Ishida R, Watanabe Y, Iwanuma O, Sakivama K, Ide Y.** Expression of HGF and IGF-1 during regeneration of masseter muscle in mdx mice. *J Muscle Res Cell Motil* 31: 71–77, 2010.
29. **Hossain M, Irwin R, Baumann MJ, McCabe LR.** Hepatocyte growth factor (HGF) adsorption kinetics and enhancement of osteoblast differentiation on hydroxyapatite surfaces. *Biomaterials* 26: 2595–2602, 2005.
30. **Huijing PA, Jaspers RT.** Adaptation of muscle size and myofascial force transmission: a review and some new experimental results. *Scand J Med Sci Sports* 15: 349–380, 2005.
31. **Iida K, Itoh E, Kim DS, Rincon JP, Coschigano KT, Kopchick JJ, Thorner MO.** Muscle mechano growth factor is preferentially induced by growth hormone-deficient *lit/lit* mice. *J Physiol* 560: 341–349, 2004.
32. **Jacquemin V, Butler-Browne GS, Furling D, Mouly V.** IL-13 mediates the recruitment of reserve cells for fusion during IGF-1-induced hypertrophy of human myotubes. *J Cell Sci* 120: 670–681, 2007.
33. **Karasik D, Kiel DP.** Evidence for pleiotropic factors in genetics of the musculoskeletal system. *Bone* 46: 1226–1237, 2010.
34. **Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF.** Establishment of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Res* 12: 2014–2023, 1997.
35. **Klein-Nulend J, Burger EH, Semeins CM, Raisz LG, Pilbeam CC.** Pulsating fluid flow stimulates prostaglandin release and inducible prostaglandin G/H synthase mRNA expression in primary mouse bone cells. *J Bone Miner Res* 12: 45–51, 1997.
36. **Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH.** Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts—correlation with prostaglandin upregulation. *Biochem Biophys Res Commun* 217: 640–648, 1995.
37. **Kondo H, Nifuji A, Takeda S, Ezura Y, Rittling SR, Denhardt DT, Nakashima K, Karsenty G, Noda M.** Unloading induces osteoblastic cell suppression and osteoclastic cell activation to lead to bone loss via sympathetic nervous system. *J Biol Chem* 280: 30192–30200, 2005.
38. **Lalani R, Bhasin S, Byhower F, Tarnuzzer R, Grant M, Shen R, Asa S, Ezzat S, Ganzalez-Cadavid NF.** Myostatin and insulin-like growth

factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight. *J Endocrinol* 167: 417–428, 2000.

39. **Leick L, Hellsten Y, Fentz J, Lyngby SS, Wojtaszewski JFP, Hidalgo J, Pilegaard H.** PGC-1 mediates exercise-induced skeletal muscle VEGF expression in mice. *J Appl Physiol* 102: 573–581, 2007.
40. **Li SH, Guo DZ, Li B, Yin HB, Li JK, Xiang JM, Deng GZ.** The stimulatory effect of insulin-like growth factor-1 on the proliferation, differentiation, and mineralisation of osteoblastic cells from Holstein cattle. *Vet J* 179: 430–436, 2010.
41. **McCroskery S, Thomas M, Maxwell L, Sharma M, Kambadur R.** Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* 162: 1135–1147, 2003.
42. **McGarry JG, Klein-Nulend J, Mullender MG, Prendergast PJ.** A comparison of strain and fluid shear stress in stimulating bone cell responses—a computational and experimental study. *FASEB J* 19: 482–484, 2005.
43. **Middleton J, Arnott N, Walsh S, Beresford J.** Osteoblasts and osteoclasts in adult human osteophyte tissue express the mRNAs for insulin-like growth factors I and II and the type 1 IGF receptor. *Bone* 16: 287–293, 1995.
44. **Mills P, Lafrenière JF, Benabdallah BF, El Fahime el M, Tremblay JP.** A new pro-migratory activity on human myogenic precursor cells for a synthetic peptide within the E domain of the mechano growth factor. *Exp Cell Res* 313: 527–537, 2007.
45. **Mullender MG, Tan SD, Vico L, Alexandre C, Klein-Nulend J.** Differences in osteocyte density and bone histomorphometry between men and women and between healthy and osteoporotic subjects. *Calcif Tissue Int* 77: 291–296, 2005.
46. **Petrella JK, Kim JS, Cross JM, Kosek DJ, Bamman MM.** Efficacy of myonuclear addition may explain differential myofiber growth among resistance-trained young and older men and women. *Am J Physiol Endocrinol Metab* 291: E937–E946, 2006.
47. **Reijnders CMA, Bravenboer N, Tromp AM, Blankenstein MA, Lips P.** Effect of mechanical loading on insulin-like growth factor-I gene expression in rat tibia. *J Endocrinol* 192: 131–140, 2007.
48. **Rittweger J, Felsenberg D.** Recovery of muscle atrophy and bone loss from 90 days bed rest: results from a one-year follow-up. *Bone* 44: 214–224, 2009.
49. **Russell ST, Marcelle JR, Rama SV, Karen E, Stephen GE.** Hepatocyte growth factor is secreted by osteoblasts and cooperatively permits the survival of haematopoietic progenitors. *Br J Haematol* 112: 438–448, 2001.
50. **Schonau E.** The development of the skeletal system in children and the influence of muscular strength. *Horm Res* 49: 27–31, 1998.
51. **Sheehan SM, Tatsumi R, Temm-Grove CJ, Allen RE.** HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro. *Muscle Nerve* 23: 239–245, 2000.
52. **Shuto T, Sarkar G, Bronk JT, Matsui N, Bolander ME.** Osteoblasts express types I and II activin receptors during early intramembranous and endochondral bone formation. *J Bone Miner Res* 12: 403–411, 1997.
53. **Spangenburg EE, Bowles DK, Booth FW.** Insulin-like growth factor-induced transcriptional activity of the skeletal alpha-actin gene is regulated by signaling mechanisms linked to voltage-gated calcium channels during myoblast differentiation. *Endocrinology* 145: 2054–2063, 2004.
54. **Standal T, Abildgaard N, Fagerli UM, Stordal B, Hjertner O, Borset M, Sundan A.** HGF inhibits BMP-induced osteoblastogenesis: possible implications for the bone disease of multiple myeloma. *Blood* 109: 3024–3030, 2007.
55. **Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J.** Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone* 41: 745–751, 2007.
56. **Tang L, Xian C, Wang Y.** The MGF expression of osteoblasts in response to mechanical overload. *Arch Oral Biol* 51: 1080–1085, 2006.
57. **Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K.** Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab* 5: 464–475, 2007.
58. **van Wessel T, de Haan A, van der Laarse WJ, Jaspers RT.** The muscle fiber type-fiber size paradox: hypertrophy or oxidative metabolism? *Eur J Appl Physiol* 110: 665–694, 2010.
59. **Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J.** Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. *Biochem Biophys Res Commun* 348: 1082–1088, 2006.
60. **Wang Y, Nishida S, Elalieh HZ, Long RK, Halloran BP, Bikle DD.** Role of IGF-I signaling in regulating osteoclastogenesis. *J Bone Miner Res* 21: 1350–1358, 2006.
61. **Wang Y, Wan C, Gilbert SR, Clemens TL.** Oxygen sensing and osteogenesis. *Ann NY Acad Sci* 1117: 1–11, 2007.
62. **Weinbaum S, Cowin SC, Zeng Y.** A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. *J Biomech* 27: 339–360, 1994.
63. **Wozniak AC, Anderson JE.** Nitric oxide-dependence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. *Dev Dyn* 236: 240–250, 2007.
64. **Yang Q, McHugh KP, Patntirapong S, Gu X, Wunderlich L, Hauschka PV.** VEGF enhancement of osteoclast survival and bone resorption involves VEGF receptor-2 signaling and beta3-integrin. *Matrix Biol* 27: 589–599, 2008.
65. **Yang SY, Goldspink G.** Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. *FEBS Lett* 522: 156–160, 2002.
66. **You L, Cowin S, Schaffler M, Weinbaum S.** A model for strain amplification in the actin cytoskeleton of osteocytes due to fluid drag on pericellular matrix. *J Biomech* 34: 1375–1386, 2001.
67. **Zaman G, Saxon LK, Sunters A, Hilton H, Underhill P, Williams D, Price JS, Lanyon LE.** Loading-related regulation of gene expression in bone in the contexts of estrogen deficiency, lack of estrogen receptor alpha and disuse. *Bone* 46: 628–642, 2010.