Physical Stimulations for Bone and Cartilage Regeneration

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Abstract

A wide range of techniques and methods are actively invented by clinicians and scientists who are dedicated to the field of musculoskeletal tissue regeneration. Biological, chemical, and physiological factors, which play key roles in musculoskeletal tissue development, have been extensively explored. However, physical stimulation is increasingly showing extreme importance in the processes of osteogenic and chondrogenic differentiation, proliferation and maturation through defined dose parameters including mode, frequency, magnitude, and duration of stimuli. Studies have shown manipulation of physical microenvironment is an indispensable strategy for the repair and regeneration of bone and cartilage, and biophysical cues could profoundly promote their regeneration. In this article, we review recent literature on utilization of physical stimulation, such as mechanical forces (cyclic strain, fluid shear stress, etc.), electrical and magnetic fields, ultrasound, shock waves, substrate stimuli, etc., to promote the repair and regeneration of bone and cartilage tissue. Emphasis is placed on the mechanism of cellular response and the potential clinical usage of these stimulations for bone and cartilage regeneration.

Keywords

Bone and cartilage regeneration; fracture repair; physical stimulation; electrical and magnetic fields; mechanical forces; ultrasound; shock waves

1 Introduction

Classical tissue regenerative engineering is an interdisciplinary field of advanced material science, cell biology, and developmental biology, with the aim of promoting the regeneration of complex tissues and organs [1]. In this process, natural or synthetic scaffolds, cells, and growth factors combine to form a construct, structurally, functionally, and mechanically similar to the native tissue that requires repair [2]. It is well known that bone disorders such as osteoporosis, bone fractures, and cartilage disease, like osteoarthritis, commonly occur due to abnormal physiology or physical injury. Several techniques and strategies have emerged to promote their regeneration. For example, guided bone regeneration (GBR) has been widely utilized as a simple therapeutic technique for effective bone reconstruction [3–5]. Autologous chondrocyte implantation (ACI) and human mesenchymal stem cell (hMSC) based treatments are promising strategies for cartilage regeneration. However, for bony

defects and cartilage degeneration, reconstructing tissues with sufficient mechanical strength and native-tissue-like function is one of the major challenges. In bone regeneration, a common obstacle is fibrous connective tissue rapidly occupying the bony defect rather than normal bone formation (osteogenesis) occurring. The resulting fibrous connective tissue buildup, with its low mechanical strength and cartilage-like structure, creates defective bone. In cell-based cartilage regenerative therapies, both ACI and hMSCs-based treatments have shown critical drawbacks for clinical use. Dedifferentiated chondrocytes form fibrocartilage instead of hyaline cartilage after transplantation in ACI and endochondral ossification following hypertrophic differentiation of hMSCs frequently occurs in hMSCs-based osteoarthritis treatments [6–12].

To address these issues, people have extensively researched biochemical stimuli including plateletrich plasma, novel biomaterial scaffolds, and various growth factors; however, most attention was put into the chemical and biological behaviors [13–17]. Since bone and cartilage are exposed to multiple internal and external physical forces, biomechanical environment plays an important role in maintaining, repairing, and remodeling their respective tissues to meet functional demands and maintain the tissue homeostasis. In fact, the physical properties of the cell micro-environment are equally important as the biochemical properties. For example, it was shown that altering the stiffness of the extracellular matrix (ECM) could direct stem cell differentiation, with increasing stiffness directing differentiation towards more mechanically competent tissues, such as cartilage and bone, and away from the more delicate adipose and neuronal tissues [18].

Physical stimuli (cyclic strain, electricity, electromagnetism, ultrasound, shock wave and laser) have already shown active roles in bone and cartilage regeneration *in vitro* and *in vivo* [19–23]. In cell-based musculoskeletal tissue engineering, these physical stimuli (**Fig. 1**) have been found to induce hMSC proliferation, modulate their behaviors, and support their differentiation by modulating their intracellular signaling pathways. This suggests that the use of such stimuli can be a promising strategy to improve bone fracture healing and cartilage regeneration. To date, some physical manipulations have already been introduced into clinical applications for bone and cartilage regeneration. The objective of this review is to identify the main physical stimulation methods that have been utilized in bone and cartilage repair and elucidate possible mechanisms of cellular response.

2   Physical stimulation for bone regeneration and fracture healing

2.1   Mechanical forces

It is well known that both extrinsic and intrinsic mechanical forces can induce tissue resistance and adaptation. The induced tissue forces are transmitted to the micromechanical environment of resident cells and thus influence the intracellular forces. Cells can subsequently modify their micromechanical environments via cytoskeletal rearrangement or molecular cascade transduction activation. This ultimately alters synthesis or degradation of the extracellular matrix, and feeds back to alter cellular sensitivity to incoming mechanical forces [24]. Studies have demonstrated that appropriate mechanical forces are important for bone cell localization, orientation, metabolism, and homeostasis [25]. The most common mechanical forces that benefit this are cyclic strain and fluid shear stress.

2.1.1   Cyclic strain—Cycles of loading and unloading cause the compression and relaxation of the extracellular matrix (ECM), which induce strain on the cells of bone or cartilage. Cyclic strain includes repeated tensile strain as well as cyclic compressive strain. Cartilage and bone are constantly exposed to cyclic strain when an individual body is moving in daily life. Tensile strain is clinically used for bone engineering in distraction osteogenesis (a surgical procedure used to repair bone by creating a fracture between two bone segments, then moving the segments slowly apart from each other). The magnitude of tensile strain is important in bone development, and in the fate determination of MSCs. For example, the magnitude of tensile strain was reportedly related to inhibition of adipogenesis (a process balancing osteogenesis and chondrogenesis) and the introduction of interruptions (rests) in strain application showed no significant effect [26]. The equi-biaxial cyclic tensile strain significantly reduced adipogenesis in mouse adipose-derived mesenchymal stem cells (ASCs) [27]. Studies have shown cyclic strain could increase bone-to-adipose ratio via Wnt pathways and upregulate the expression of paladin (an actin-associated protein), to promote the osteogenesis. Stretch-activated cation channels may also contribute to osteogenesis [28]. Forces acting on cells may change protein conformation and thus expose the binding sites in a functionally relevant way [29]. Elements of the cytoskeleton bridging actin fibers (including lamin proteins) to the nuclear membrane also show an important role in osteogenesis during this mechanical stimulation [29].

In addition, the piezoelectric properties of bone make it generate electricity in response to mechanical stress. The amplitude of the electrical potential generated in stressed bone is determined by the rate and magnitude of the applied load and the resulting bone deformation. The electrical polarity is dependent upon the directions of loading and bending. Normally, when bone is bent, the concave sides (under compression) become negatively charged and the convex sides (under tensile) become positively charged, which make the bone grow more on the compressive side and degrade more on the stretched side [30]. In this case, the mechanical stress is also capable of stimulating bone regeneration through electrical induced pathways, the mechanism of which is detailed in the following electrical stimulation section.

2.1.2   Fluid shear stress—The circulatory system (e.g. flow of blood) also produces pulsatile or oscillating shear stress on musculoskeletal cells. The shear stress induced by fluid flow plays a significant role in bone development, especially in the osteogenic differentiation process of stem cells. Studies have shown the application of both continuous flow and pulsating fluid flow (PFF) to increase osteogenic differentiation of ASCs as compared to static cultures. The greatest osteogenic induction was seen with PFF. Tjabringa *et al.* state that 3 hours after PFF application, gene expression of Runx2 was increased, while that of osteopontin (OPN) did not change, suggesting PFF may affect the early stages, but not the late stages of osteogenic differentiation. This is because Runx2 expression is an indicator of early osteogenesis and OPN expression is an indicator of late osteogenesis. The enhancement of osteogenesis from fluid flow may relate to the distribution of nutrient and growth factors in the cell. Fröhlich *et al.* found enhanced expression of bone-specific markers in perfusion cultures with uniform distribution, as compared with static culture only

present at the outer regions in static culture [31]. Thus, the improved osteogenesis from fluid flow may be attributed to the better distribution of nutrients and growth factors.

The flow stimulation of ASC osteogenesis may be explained by an indirect mechanism via polyamines, the enzyme Cox-2, and nitric oxide (NO). Studies have shown PFF to increase the gene expression of spermidine/spermine N (1)-acetyltransferase (SSAT), an enzyme associated with polyamine activity [32]. Higher intracellular calcium activity may also be involved in shearinduced osteogenesis through PKC and ERK 1/2 pathways, downstream of NO production, in PFF activated ASCs osteogenesis [33]. Fluid shear stress can also upregulate the expression of integrin α5β1, which has been identified as an important factor in promoting osteogenesis through ERK 1/2 activation [33]. ERK activation is proven to be important in determining osteoblast survival, proliferation, and differentiation [34].

2.1.3 The molecular mechanism of mechanical force transduction—Once the cell has detected a local mechanical stimulus, the signal needs to be converted into a biochemical response. For the general pattern of mechanical force sensing machinery in the musculoskeletal system, the ECM-integrin-cytoskeletal signaling axis has gained the most attention (Fig. 2). Transmembrane receptors called integrins connect the ECM to intracellular cytoskeleton elements consisting of actin filaments, non-muscle myosin, and associated proteins [35]. The cytoskeleton achieves structural cohesion by creating a dynamic balance between the counteracting forces of compression and tension [36]. The force-induced conformational changes of the cytoskeleton directly alter chromatin structure and thus modulate gene transcriptional activity. This occurs via direct connections of cytoskeletal elements to DNA [37] or by activating integrin-mediated intracellular pathways that involve focal adhesion kinases (FAKs) or Src tyrosine kinases [38]. Neighboring cells that are attached to the affected cell via cadherincontaining adhesion complexes could be mechanically transferred, accordingly inducing molecular changes [39].

On the other hand, integrin-mediated transmission of membrane strain induces activation of Akt, resulting in downstream activation of both β-catenin and Ras homolog gene family member A (RhoA). This increases cell stiffness, which results in repression of adipogenic genes [38]. As an effect of force, calcium influx is frequently regulated by voltage-sensitive calcium channels (VSCC). These channels are partially anchored in the cell membrane and thus, are capable of attaching to the ECM and responding to mechanical stimulation in osteoblasts [40]. *In vitro* inhibition of T type VSCC significantly reduces the expression of both early and late mechanoresponsive genes in osteoblasts [41]. However, the mechanism of mechanical force transduction is complex and not clear, warranting further investigation.

2.2 Electrical and electromagnetic stimulation

2.2.1 Electrical stimulation (ES)—Physiological electric fields (EFs) serve as an efficient tool to control and adjust the cellular and tissue homeostasis. The human body generates a biological EF ranging between 10 and 60 mV at various locations [42]. Bioelectricity is very important in the wound healing process. When a wound is created, a steady direct current (DC) EF is initiated. This endogenous EF guides cell migration toward the wound edge. On the contrary, wound healing is compromised when the EF is inhibited

[43]. In 1953, Yasuda *et al.* applied continuous electrical current to a rabbit femur for 3 weeks and demonstrated new bone formation around the cathode [44]. Since then, use of EFs for bone healing applications has been widely researched [45]. Capacitive coupling electric field (CCEF) and inductive coupling electromagnetic field (EMF) are also being used more frequently in recent years. Both DC and alternating current (AC) have been observed to enhance osteogenesis when cells at the cathode are stimulated with a current of 5–100 μA [46].

Electrical potentials have been proven to play an important role in bone cell proliferation, migration, and remodeling both *in vitro* and *in vivo* [47, 48]. Some implant materials, such as electrically active ceramics, including polarized hydroxyapatite (HA), and piezoelectric ceramics, which produce an electrical potential under mechanical loading, have been found to induce bone ingrowth and improve bone formation around implants respectively. The mechanism by which electrical activity influences biological responses is likely to result from preferential adsorption of proteins and ions onto the charged surface. Numerous studies have emphasized the importance of surface charge species on cell behavior at the biomaterial interface [49, 50, 47]. In calvarial bones of rats, after implanting electrically polarized HA plates, improved bone ingrowth and enhanced osteoblast activity were observed, with complete bone penetration into polarized implants occurring as early as 3 weeks [47]. In this study, the bone formation increase that occurred on the negatively charged surfaces (N-surfaces) of the polarized implants was likely due to accumulating Ca^{2+} ions on the surfaces. Molecules such as fibronectin, osteocalcin, and bone morphogenetic proteins (BMPs), on the other hand, adhere to the positively charged surfaces (P-surfaces) to improve osteoblast migration [47]. Nakamura *et al.* also observed the surface charge of polarized HA influencing protein adsorption onto the HA surface and thus enhancing the osteoconductivity of HA. Fibrin was presented as a key protein in the early stages of osteoconduction. Its adsorption was accelerated on both N-surfaces and P-surfaces through ionic and pH changes via attracting calcium ions and –COOH groups of fibrin respectively. Specifically, –COOH groups of fibrin were attracted to the P-surfaces, while calcium ions were attracted to the N-surfaces. The resulting positively charged ion layer further encouraged fibrin adsorption (**Fig. 3**). Subsequently, a network scaffold is formed by absorbed fibrin, platelets, and osseous cells. After adhesion to the fibrin on the P-surface occurs through integrin $α_{2b}β_3$, the activated platelets further release a variety of growth factors that stimulate the osseous cells. The coagulation components played an important role in the early stages of osteoconduction [49]. In addition, hyaluronan, an extracellular matrix component, also plays a key role in the cellular interactions with charged surfaces. The negatively charged surface of osteoblasts that hyaluronan induced has been shown to mediate initial contact between cell and metal surfaces [51].

The exact mechanism underlying the intracellular signal transduction of ES in bone repair is still unclear. Several hypotheses are shown in **Fig. 2**. 1) External EFs could alter the ion flux via cell membrane proteins (such as ion channels, transporters, pumps, and enzymes) and subsequently lead to an ion concentration (such as Ca^{2+}, Na^+, Cl^- and K^+) change, which may cause a depolarization of excitable cells and trigger the related cellular signaling [52]. For example, ES could activate the phosphatidylinositol-3-kinase (PI3K) and mammalian

target of rapamycin (mTOR) pathways, which lead to the transcription of transforming growth factor-β (TGF-β) family factors such as BMP-4. 2) Applied current could change the cell gap junctions, which affect the exchange of certain signaling molecules such as calcium, cyclic nucleotides, and inositol phosphates. Much evidence indicates that gap junction communication is necessary for the development and maintenance of a differentiated osteoblast phenotype, including the production of alkaline phosphatase, osteocalcin (OCN), bone sialoprotein, and collagen [53]. 3) EFs may also affect ligand-receptor binding by changing the conformation or expression of receptors. For example, EFs could increase the expression of adenosine A2A receptors (A_{2A} Rs) or integrin β molecules, both of which influence their related intracellular pathways with roles in antiinflammatory and differentiation processes [54]. 4) EFs may also stimulate higher metabolic activity, which could induce intracellular ATP depletion and thus alter the membrane characteristics such as endo- and exocytosis, adhesion, and motility [55]. 5) EFs could change ECM compositions by affecting the ECM components including soluble ions and charged groups in glycosaminoglycans (GAGs) and proteins [56].

2.2.2 Electromagnetic stimulation—Pulsed electromagnetic fields (PEMFs), which are generated from an unsteady current being passed through a coil, have been approved by the FDA to treat nonunions of bone fractures and related problems since 1979 [57]. Under PEMF stimulation, osteoblasts were found to exhibit increased osteogenesis caused by elevated expression of TGF-β1 [58] and BMP-2/4 [59] and reinforced intracellular calcium transients [60]. In an ovariectomized rat model, PEMFs were found to prevent ovariectomy-induced bone loss through activation of the Wnt/ β-catenin signaling pathway [61]. In an identical model, long-term PEMF stimulation treatment alleviated lumbar vertebral osteoporosis by increasing bone formation and suppressing bone resorption through regulation of the Wnt3a/LRP5/ß-catenin and OPG/RANKL/RANK signaling pathways [62]. Ehnert *et al.* identified a specific extremely low-frequency pulsed electromagnetic field (ELFPEMF) (10 to 90.6 Hz) that supports human osteoblast function in an ERK1/2-dependent manner. The ELF-PEMF by producing non-toxic amounts of reactive oxygen species (ROS), induced antioxidative defense mechanisms in these cells [63, 64].

In bone tissue engineering, PEMFs were found to modulate the cell cycle of MSCs of different origins and enhance their differentiation and proliferation. This could be seen by their enhanced production of ECM and growth/differentiation factors including TGF-β and BMPs [23, 65]. A wide range of electromagnetic stimulation frequencies (between 2 and 123 Hz) has been shown to be effective in improving osteogenic stimulation of ASCs [66], characterized by increased intracellular calcium and Alizarin Red S staining after 14 days induction [67]. The stimulation increased alkaline phosphatase activity and cytoskeleton tension. It also induced higher expression of ALP, OPN, collagen type I (Col I), and Runx2 after 21 days induction [66]. PEMFs were also used as an adjuvant element in many studies, along with osteo-inductive medium. However, which PEMF parameters (dose, frequency, and intensity) enable the most optimal repair in a clinical setting is still an unanswered question.

Similar to the mechanism of mechanical stress, that of PEMFs on bone regeneration is more complicated than initially expected [68]. PEMFs may play roles through 1) changing the

physical and chemical properties of a cell membrane by altering the ion flux and membrane potentials [69, 70]; 2) affecting the assembly and arrangement of the actin cytoskeleton; 3) modulating the intracellular Wnt/ β-catenin and TGF-β/BMP signaling pathways, leading to upregulated expression of key cytokines such as TGF-β1 and BMP 2/4 [59, 58, 61]; 4) regulating the oxidative state of cell [71, 72]. However, the precise cellular mechanism is still unclear. More mechanistic investigations are needed.

2.3 Ultrasound

Ultrasound (US) usually refers to a longitudinal wave propagation, a special type of sonic wave with a frequency greater than 20 kHz (this is the upper limit of human audibility), that causes local oscillation of particles. Ultrasound with a frequency around 3~10 MHz is widely used in clinical settings for diagnostic and therapeutic purposes. It is also one of the well-established therapeutic physical stimuli for bone healing. Since ultrasound was first reported to stimulate bone healing in 1950 [73], numerous efforts have been spent over the past several decades to prove its therapeutic effects in animal models [74, 75]. In particular, low intensity pulsed ultrasound stimulation (LIPUS), using intensities less than 50 mW/cm^2, was reported to improve ECM synthesis, accelerate bone healing, and reactivate failed healing processes [76, 74]. The use of ultrasound in improving bone regeneration was recently approved by the FDA for human application. Ultrasound-based non-viral gene delivery was also recently found to induce bone formation *in vivo* [77]. In *in vitro* cell studies, ultrasound was found to enhance the expression of osteoblasts maturation markers, such as OCN, bone sialoprotein (BSP), and Ca^{2+} [78–83]. With treatment of LIPUS, the expression of chemokines such as monocyte-chemoattractant proteins (MCP)-1, macrophage-inflammatory proteins (MIP)-1, and receptor-activator of nuclear factor kappa-B ligands (RANKL) is enhanced, and mechanoreceptor angiotensin II type I receptor (ATI) is activated on the surfaces of osteoblasts [84]. Under LIPUS exposure, the production of NO and prostaglandin E2 (PGE2) was up-regulated. The former is a free radical gas involved in the regulation of vascular endothelial growth factor (VEGF) expression and is important to bone formation [85]; the latter is an arachidonic acid-derived metabolite, associated with bone formation and resorption. In differentiating murine osteoblasts, LIPUS has been found to enhance RANKL gene expression 10-fold compared to unstimulated controls after 3 weeks of LIPUS treatment [84]. It was also reported that LIPUS combined with growth factors such as calcium-regulating hormones, 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) [86], BMP2 [87], or BMP7 [88], can stimulate bone repair.

LIPUS-induced bone healing can be influenced by the processes of 1) inflammation, 2) soft callus formation, 3) angiogenesis, 4) early osteogenesis, 5) bone formation, and 6) bone remodeling [89]. There are several theories to illustrate these mechanisms (**Fig. 2**). In the first theory, oscillatory displacement of the cell membrane caused by the ultrasound wave triggers oscillatory displacement between intracellular elements of different densities [90]. The very low strains induced by the ultrasound on cells *in vitro* have been reported to induce a prompt fluidization of the cytoskeleton together with acceleration of cytoskeletal remodeling events [91]. The second theory is the bilayer sonophore model. In this model, ultrasound application periodically pulls the two lipid layers apart and back, leading to intramembranous hydrophobic spaces expanding and contracting accordingly [92]. In the

third theory, integrins play a key role in converting LIPUS signals into biochemical responses [93]. The type of integrins including α2, α5, β1, and β3 integrins, varies in response to different cell origins [94, 80, 95]. In the fourth theory, ultrasound induces intracellular stress and strain which are maximized within the cell at two distinct resonant frequencies. Stimulated load-inducible gene expression therefore is maximized when the excitation frequency matches the cell's resonant frequency [96]. The fifth theory is related to P2Y receptor activation. P2Y receptors are G protein-coupled receptors (GPCRs) that are activated by adenine and uridine nucleotides and nucleotide sugars. Studies have shown LIPUS treatment to induce osteoblastogenesis by releasing purines, such as ATP, and activating P2Y receptors[97]. The sixth theory involves calcium signaling regulation. Ca^{2+} signals are oscillatory and these signals (also generated via the RhoA GTPase pathway) are crucial for bone marrow-derived mesenchymal stem cells (BMSCs) differentiation [98]. Finally, the last theory is about the connexins mediated gap junction. Studies have shown that gap junctions are essential for LIPUS's effect on osteogenic differentiation of MSCs [99, 83].

In addition, ultrasound can modulate the micro-environment by heating, cavitation, acoustic streaming, or triggering delivery of growth factors to engineered cells [100]. The physical effects of LIPUS that induce biological responses can be divided into thermal and non-thermal categories. Temperature increases can regulate thermo-sensitive enzymes like metalloproteinase, which are important for bone matrix remodeling. Non-thermal effects include oscillatory strains induced by ultrasonic waves (which can directly affect the mechanosensitive elements at very high frequencies), acoustic radiation forces (resulting in a low-frequency cyclic mechanical stimulus), strain gradients, and fluid-flow (such as acoustic streaming and microstreaming). Radiation force, fluid flow, and strain gradients can create shear stresses on cell membranes. Acoustic streaming and microstreaming can play important roles *in vitro.* The former results in nutrients redistribution via improved circulation of molecules in the culture medium or via increased fluid flow *in vivo* [101]; the latter is generated in response to oscillating gas bubbles or other small acoustic in homogeneities and causes circulatory movement of fluid [100].

The involved pathways in US treatment are complex. In murine MC3T3-E1 pre-osteoblasts, COX-2 expression, which is important for PGE2 production, was regulated via FAK and mitogenactivated protein kinases (MAPKs), Erk1/2, PI3K, and Akt kinase signaling in response to ultrasound treatment [94]. The expression of iNOS (in charge of the NO production) was found to be induced by ultrasound through the canonical NF-kB pathway which is preceded by activation of Ras, Raf-1, MEK, Erk, and IKKα/β kinases [102]. LIPUS induced p38 MAPKs and Erk1/2 MAPKs which were found to be crucial in the process of osteogenic differentiation in human periodontal ligament cells (HPDLC) and the murine pluripotent mesenchymal cell line C2C12 [83, 103]. However, more investigations are needed to illustrate the involved signaling transduction cascade under ultrasound exposure.

2.4 Shock wave

A shock wave is a kind of short-duration, acoustic pressure wave consisting of two phases, the positive phase evoking compressive stress (peak pressure: 30–100 MPa) and the negative phase arousing tensile and shear stress (negative pressure). These waves can be produced by various generators such as electro-hydraulic, electromagnetic, piezoelectric, or pneumatic generators [104]. After propagating into tissue, shock waves may lead to micro-bubbles of liquid molecules and cavitation effects on the focal area. Shock waves were introduced to increase cell membrane permeability and facilitate the delivery of macromolecules into cells [105]. Use of shock waves, referred to as extracorporeal shock wave therapy (ESWT), is normally looked as "extracorporeal" and "non-invasive" stimulation mainly focused on the treated area. It is known that ESWT is able to relieve pain, reduce inflammation, induce neo-angiogenesis, and stimulate stem cell activities, thus improving tissue regeneration and healing. ESWT has been applied in the musculoskeletal field as orthotripsy and regenerative medicine to promote bone remodeling [106–108], restore the healing process in cases of non-unions [109], loosen the bone cement during revision arthroplasty [110], and enhance bone callus formation during bone lengthening [111]. Shock waves also can promote osteoblasts growth and differentiation as well as their expression of TGF-β1 in a dosedependent manner [112]. In osteoarthritis (OA) treatment, ESWT was used to regulate subchondral bone remodeling and improve trabecular microarchitecture. Compared to the non-treatment OA group, the ESWT-treated group showed increased an osteocyte count and a higher percentage of subchondral trabecular bone [113]. Increased proliferation and migratory capacity was also shown in human BMSCs when exposed to shock waves [114]. ASCs exposed to ESWT have shown enhanced production of osteogenic markers such as RUNX2, ALP, and mineralized matrix. However, the production of reactive oxygen species (ROS) was also increased [115]. ESWT could also affect the growth ratio of bone marrow osteoprogenitor cells to bone nodules, which is related to the induction of TGF-β1 molecules [116].

The acoustic shock wave induces tissue to absorb, reflect, refract, and propagate the mechanical pulsed energy. The mechanisms of shock waves' effects on bone healing are possibly related to the micro-fractures and cavitation they induce [117]. The micro-fractures and cavitation may trigger the initiation of remodeling cycles and neovascularization [107, 118]. Thus, they regulate the growth and maturation of osteoprogenitor cells, membrane polarization, expression of BMPs, and activation of so-called mechanotransduction pathways that are related to acoustic stimulations [106, 119–122]. During the mechanotransduction process, mechanosensory components in cell membranes such as integrins, ion channels, and various sensors and growth factor receptors may be activated by shock wave-induced forces. Several signaling pathways (e.g. MAPK-ERK pathway, P13K-Akt-iNOS pathway) may be involved in the corresponding biological events of cytoskeletal rearrangement and nuclear expression modulation [123]. ESWT can also regulate the sub-membrane reduction-oxidation (redox) reactions elicited by early O2 production for tyrosine kinase-mediated ERK activation, resulting in phosphorylation of CBFA1 (core-binding factor alpha1), the transcription factor for osteoblastic differentiation [124]. However, the overlap of several pathways and interactions between them make it more complicated to

illustrate the exact signal transduction. More investigation needs to be done to clarify the precise molecular mechanism before translating it to clinic use.

2.5 Substrate stimulus

The nature of growth surfaces, scaffold or substrate, always plays a significant role in influencing the cell behavior. Good osteoconductivity and osteogenic ability are prerequisites for scaffolds used in bone engineering to promote the new bone formation. The idea is that scaffolds should provide a good environment to guarantee secure attachment, survival, and distribution of osteogenic cells grown into or surrounding them. The substrate stimulus could directly affect the structural changes of bone or cartilage cells through integrins, focal adhesions, or the actin cytoskeleton. Indirect mechanisms via G-proteins or ion channels are also possible. In scaffoldinduced osteogenesis, increased phosphorylation by FAKs at tyrosine 397 was observed [125]. Stimulation by scaffold ions may drive osteogenesis of ASCs through an indirect mechanism in which signals are transduced through receptors, ion channels, or G-proteins to the nucleus where the expression of related genes was regulated. For example, the calcium ions could enter the cell via calcium receptors which interact with G proteins. Calcium ions have been shown to stimulate proliferation of osteoblasts and magnesium ions have been associated with increased mineralization [126]. McCullen *et al.* also showed that ionic calcium enhanced mineralization in human ASCs [127]. Additionally, nanoscale topographical features in growth substrates influence stem cell behavior [29]. Elasticity also has a plausible effect on the osteogenesis of ASCs [26, 18, 67, 128].

The area of cell adhesion onto the matrix substrate has been also shown to regulate cell behavior. For example, stem cells forced to attach on large fibronectin islands show an elongated morphology, different from the more rounded shape that occurs when attached on smaller islands. The enhanced osteogenic commitment was due to increased RhoA and Rho-associated protein kinase (ROCK) activity [129].

2.6 Other physical factors

Besides the aforementioned physical stimulations on bone regeneration, some other physical methods have also been introduced into bone regeneration and healing. Laser periodontal therapy (LPT) is a laser-based procedure developed as an effective debridement technique to treat periodontitis [130]. Low-level laser therapy (LLLT) with proper doses and output powers was also reported to stimulate cellular metabolism, increase protein synthesis, and subsequently enhance bone regeneration [131]. Temperature could rise in a surgical operation like osteotomies and the elevated temperature could disrupt the bone healing [132]. Heat generation during osteotomy is one of the important factors influencing the development of osseointegration [133]. One recent study showed ultraviolet (UV)/O_3 irradiation for 5 min significantly decontaminating H_3PO_4- modified hydroxyapatite surfaces and improving their wettability, thus facilitated osteoblast growth and function [134].

3 Physical stimulation for cartilage regeneration

3.1 Mechanical forces

Mechanical load is an important regulator of chondrocyte metabolism and is required for maintaining normal cartilage matrix properties. The natural environment of articular chondrocytes in the body is a dynamic mechanical one involving various biomechanical forces including compression, tensile stretching, shear stress, hydrostatic pressure, and osmotic stress [135]. These forces have varying origins. The direct contact between joint surfaces can produce both static and dynamic compression. Tensile loading can result from physical activities such as gymnastics. The synovial fluid in articular cavities generates shear stress. Charged proteoglycan (PG) in the cartilage matrix makes hydrostatic pressure in chondrocytes. Lastly, osmotic stress is the result of the influx and efflux of fluid within the cartilage matrix during joint loading [136, 135]. Overall, both catabolic and anabolic factors contribute to ECM synthesis and remodeling in response to mechanical stimulation [137, 138].

Extra mechanical stimuli can also influence the metabolism and gene expression patterns of normal and osteoarthritic chondrocytes. For example, chondrocytes from OA cartilage can benefit from optimized compressive stimulation by enhancing the biosynthetic activity reflected by greater ECM production. One *in vitro* study has shown short-term compressive stimulation can significantly induce aggrecan (ACAN), COL2A1, COL1A1, proteoglycan 4 (PRG4), and COL10A1 gene expression in a zone-dependent manner, while long-term compression can increase collagen type II, ACAN immunostaining, and total GAG content [139]. Dynamic compression upregulates the gene expression of ACAN and type II collagen, while static compression downregulates it in chondrocytes [140]. It is known that mechanical stimulation not only changes the biosynthesis of load-bearing ECM molecules (e.g. aggrecan and collagen type II) but also regulates articular cartilage lubrication molecules (e.g. PRG4, lubricin, superficial zone protein (SZP), etc.) [141, 142]. A high shear strain can induce extensive rearrangements of the focal adhesions and the actin cytoskeleton of the chondrocytes [143]. Cyclic hydrostatic pressure was reported to stimulate the chondrogenic differentiation of BMSCs in pellet cultures [144]. Under intermittent hydrostatic pressure, inhibited matrix metalloproteinase and pro-inflammatory mediator release was observed in human osteoarthritic chondrocytes *in vitro* [145].

Mechanical stimulation can also be exerted on MSC-seeded constructs for cartilage tissue engineering applications, as seen in **Fig. 3**. For example, dynamic compression combined with exogenous SOX-9 promotes chondrogenesis of ACSs in a poly(lactic-co-glycolic acid) (PLGA) scaffold [146]. Compressive loading upregulated the expression levels of chondrogenic genes in MSC-seeded porous hyaluronan-gelatin constructs [147]. Cyclic tensile loading enhanced PG synthesis rates in MSC-seeded collagen-PG scaffolds [148]. A combination of shear and dynamic compression leads to chondrogenesis of human MSCs [149]. Some research has shown chondrogenesis of human BMSCs in fibrin-polyurethane composites could be modulated by frequency and amplitude of dynamic compression and shear stress [150]. However, the responses of MSCs to mechanical stimulation sometime are different from those of chondrocytes. Huang *et al.* demonstrated that 21 days of compressive

loading significantly reduces the compressive mechanical properties and biochemical contents of bovine MSC-seeded agarose constructs [151]. Thorpe *et al.* recently showed that both the compressive modulus and PG content of porcine MSCseeded agarose constructs were significantly reduced in a long-term, dynamic mechanical compression application, compared to free-swelling controls [152]. It is important to highlight that mechanical loading could synergistically improve the composition and mechanical properties of neocartilage when integrated with growth factors, such as TGF-β and insulin-like growth factor-1 (IGF-1) [153].

The molecular mechanisms of mechanical signal transduction in chondrocytes are complex and not fully understood. The physical properties of chondrocytes are related to this mechanical force transduction. For example, the pericellular matrix (PCM) of chondrocytes was proven to transfer forces between the cell cytoskeleton and its ECM. The viscoelasticity of chondrocytes, which is determined by the integrity and organization of their actin filaments and intermediate filaments, is also very important in cartilage's response to the mechanical force. Mismatches in viscoelastic properties could result in further cartilage degradation due to the disparities between neocartilage and adjacent tissues. The actin and intermediate filaments bear the cytoskeletal tension and the microtubules serve as struts to resist compression. Chondrocytes could respond to mechanical stimuli by remodeling their actin cytoskeleton, which is linked to the ECM through focal adhesions. These focal adhesions transfer the signal of external physical forces into the appropriate biochemical events in a Rho kinase-dependent manner [154]. Specifically, the Rho GTPases activate ROCKs, which phosphorylate and activate Lim kinases, which in turn phosphorylate and inhibit the actin-depolymerizing protein, cofilin. Studies have shown the actin cytoskeleton was changed by dynamic compression acting on agarose-embedded chondrocytes. Rho kinase activity is required for this actin reorganization and the change in gene expression that occurs [154]. Chondrocyte nuclei and their nucleoskeletons also play a vital role in mechnotransduction. A 15% compressive strain greatly decreases the height and volume of the chondrocyte and its nucleus. The actin cytoskeleton plays an important role in this deformation behavior [155]. The signal transduction mechanisms of these force-induced biochemical responses are still obscure. There are several pathways involved as shown in **Fig. 4**.

1) Integrin signaling.—Fibronectin and integrin receptors provide the vital connection between the ECM and the cytoskeleton [156] involved in cell adhesion, ECM remodeling, and chondrocyte metabolism [157]. Studies have shown that human articular chondrocytes use α5β1 integrins as mechanoreceptors. Stimulation of this integrin modulates ion channels, the actin cytoskeleton, focal adhesion, paxillin, and β-catenins. As a result, IL-4 is secreted in an autocrine manner via Type II receptors, to induce membrane hyperpolarization, increase levels of aggrecan, and decrease matrix metalloproteinase 3 expression. However, healthy chondrocytes show a different response than osteoarthritic cartilage [158].

2) Purinergic signaling.—Extracellular purines, such as ATP, adenosine, and pyrimidine, act as extracellular signaling molecules, activate related purinergic receptors

[159]. ATP is involved in the mechanotransduction signaling cascade in chondrocytes by paracrine, upregulate matrix production, and decrease MMP 3 activity after mechanical stimulation. The purinergic pathway that ATP induces activates intracellular Ca^{2+} signaling [160, 161].

3) Calcium signaling.—The calcium signaling includes the intracellular PLC-inositol 1,4,5- trisphosphate pathway, stretch-activated ion channels, and the transient receptor potential vanilloid 4 (TRPV4) pathway. Under mechanical press, an influx of Ca^{2+} could induce a wave of Ca^{2+} uptake through mechanosensitive ion channels from the extracellular medium. The Ca^{2+} concentration transiently increasing in the intracellular site may be one of the earliest events in the response of chondrocytes to mechanical stimulation [162]. TRPV4 not only regulates SOX9 expression, but it also mediates the response to osmotic stress, especially for hypoosmotic stress [163, 164]. Through Src kinases, Ca^{2+} also regulates the integrin-mediated signaling pathway, converged on ERKMAPK by a single application of cyclic compression (1kPa, 1Hz, 30min). Mechanical stimuli including compression, fluid flow, hydrostatic pressure, and osmotic stress can influence Ca^{2+} signaling in chondrocytes [161, 165–170].

4) MAPK/ERK pathway.—Several studies have shown downstream activation of MAPK pathways when chondrocytes were subjected to mechanical stimulation. MAPK signaling was found to be force-dependent in intact cartilage. Mechanical compression activated ERK1/2, JNK, and p38 pathways by stimulating phosphorylation in distinct temporal patterns [171]. Shear- and compression-induced chondrocyte transcription require MAPK activation in cartilage explants [172]. One microarray study has shown that hyperosmotic stress leads to regulation of a wide variety of genes, which involves transduction through p38 MAPK and ERK1/2 pathways [173].

5) TGF-β signaling.—TGF-β signaling has been shown to be involved in the responses of chondrocytes and MSCs to mechanical stimuli. For example, dynamic compression transiently activates Smad2/3 in chondrocyte-agarose constructs [174] and bovine bone marrow stromal constructs during chondrogenesis [175]. Mechanical load promotes chondrogenesis of hMSCs through the TGF-β pathway by up-regulating TGF-β gene expression and protein synthesis [176].

3.2 Electrical stimulation

The electrochemical properties of articular cartilage occur from the electrically charged nature of the tissue. Electric potentials develop in cartilage by the flow of charged particles across negatively charged PG in and out of the ECM [177, 178]. Applying an external electric potential or current to cartilage can produce stress and deformation in the tissue [178]. It has been hypothesized that the electric fields associated with the dynamic loading of cartilage may affect its growth, remodeling, and biosynthesis [179]. Two modes of electrical stimulation are commonly utilized: (1) direct current (DC) and (2) capacitive coupling (CC). DC (5 mA) was reported to stimulate the differentiation of MSCs into chondrocytes and enhance the proliferation of differentiated chondrocytes [180]. Chondrocytes exhibiting cathodal migration when subjected to applied DC electric fields has

also been reported [181, 182]. As early as 1978, the effect of a capacitively coupled electric field (CCEF) on chondrocyte DNA synthesis via Na^+ and Ca^{2+} fluxes was reported [183]. Selective capacitively coupled electrical signals could upregulate the gene expression of cartilage matrix proteins (e.g. ACAN and type II collagen), in which the duration, response time, amplitude, duty cycle, and frequency could affect matrix production [184–186]. Even in the presence of interleukin (IL)-lβ, a defined capacitively-induced electrical signal could result in significant upregulation of cartilage matrix proteins while significantly attenuating the upregulation of MMPs in full-thickness osteoarthritic adult human articular cartilage explants [186].

However, how the electrical signals transform and influence cell behavior is still unclear. There are several pathways that are potentially involved: 1) Adenosine receptors. Evidence has shown that adenosine receptors have been implicated in the electrotransduction process for cartilage [187]. Stimulation of both the high-affinity A2a and low-affinity A2b adenosine receptors resulted in elevated cyclic AMP and subsequent activation of anti-inflammatory pathways via protein kinase A (PKA) and exchange protein-activated directly by cyclic AMP (EPAC). This in turn lead to the suppression of NO and PGE2 and downstream feedback inhibition of tumor necrosis factor (TNF)-α and IL-lβ [188]. 2) Calcium signaling. Studies have shown CCEF (60 kHz, 20 mV/cm) to increase the expression of cartilage matrix protein genes and suppress the expression of the MMP gene in bovine articular chondrocytes. This occurs by an influx of Ca^{2+} through voltagegated calcium channels rather than from intracellular Ca^{2+} repositories, and has downstream consequences on calmodulin, calcineurin, and nuclear factor of activated T-cells (NF-AT) rather than on phospholipase C and IP(3) [189]. The transduction pathway of pulsed CCEF stimulates human chondrocyte proliferation, which involves calcium, calmodulin, cGMP, and nitric oxide synthase [190].

3.3 Magnetic stimulation

There are two modes of magnetic stimulation: (1) Static magnetic fields (SMFs), which are generated from the material properties of a permanent magnet; and (2) PEMF. It is well established that cellular responses to magnetic stimulation depend on the intensity, frequency, type of field (static or oscillatory), waveform (sinusoidal, square, etc.), cell status (precursor or differentiated), and type of cell exposed [191, 192]. In *in vitro* human chondrocyte cultures, 0.6 T SMFs significantly increase chondrocyte proliferation and viability [193]. Additionally, 0.4 T SMFs were also reported to promote PG synthesis of primary bovine and human articular chondrocytes and potentiate the chondrogenic differentiation of human bone marrow stromal. The differentiation was synergistically augmented in the presence of TGF-β3 [194]. However, there have been relatively few studies on the effect of SMFs on chondrocytes, compared to using PEMFs. PEMFs have been clinically investigated in OA patients with encouraging results [195, 196]. In *in vitro* studies, PEMFs have been found to cause a number of physiological effects in both monolayer chondrocyte cultures and tissue explant models. These benefits include enhanced proliferation [197–199], anabolic activities, PG synthesis [200–203] and anti-inflammatory responses [187, 204]. After surgical implantation of neo-cartilage *in vivo*, PEMFs prevent the catabolic effects of inflammation by upregulation of A2A receptors. The combination of

EMFs and IGFs has been reported *in vivo* to provide a more chondro-protective effect than any single treatment alone [201]. In stem cell-based tissue engineering for chondrogenesis, studies both *in vitro* and *in vivo* have shown that EMF exposure may promote chondrogenic differentiation [205, 206], increase ECM component synthesis, and control the inflammatory events, all while downregulating the expression of inflammatory mediators such as IL-1 and MMPs [22, 207–209].

As for the mechanisms of magnetic stimulation, studies have shown that MFs (e.g. PEMFs) alter ion channels, ligand-binding sites, and receptor density and distribution in the cell membrane, consequently affecting transmembrane signaling [210]. To date, there is no identified magneto receptor or even a magnetotransduciton theory to clarify the cellular responses to MF stimulation. Lorentz force that is produced by the movement of charged ions in an MF may play a key role in the biological behaviors that MFs induce. Several intracellular molecular signaling pathways are involved in the stimulation. 1) Calcium signaling. Two studies have shown the involvement of the Ca^{2+} signaling pathway in chondrogenesis under the stimulation of MFs. One study has shown strong MFs (3 T) significantly increase the intracellular Ca^{2+} concentration after 6 hours of exposure and cause deleterious effects on human chondrocytes [211]. Another study has shown moderate strength (0.4 T) SMFs induce chondrogenic differentiation of human BMSCs and increases intracellular Ca^{2+} within 20 seconds of exposure [194]. 2) TGF-β signaling. The enhancement of chondrogenesis by a PEMF is associated with an increase in TGF-β1 synthesis, mediated by binding of AP-1, which may be modulated by phosphorylation of JNK. Moderatestrength magnetic fields (0.4 T) can induce chondrogenesis of BMSCs by upregulating the expression of the cartilage maker gene SOX9, type II collagen, and aggrecan through a TGF-β- dependent pathway. SMFs alone cause TGF-ß secretion in BMSCs chondrogenic culture and this effects of SMFs could be abrogated by the TGF-β receptor blocker SB-431542 [194]. 3) Adenosine $A_{2a}R$ mediated pathway. PEMFs have been shown to reduce the concentration of TNFα and IL-1β via the up-regulation of $A_{2a}R$ and regulation of the NF-κB pathway [212, 188]. 4) MAPK signaling. Hsieh *et al.* found that strong (3 T) MFs upregulate the phosphorylation of ERK1 and ERK2 after 8 to 96-hour exposure times [211].

3.4 Ultrasound

LIPUS, as a non-invasive, efficient, and cost-effective method of improving the healing of osteochondral defects [213] and is a good supporter of cartilage regeneration [214]. These effects of US are showed by enhancing the viability of cells, matrix protein synthesis, and matrix integrity with no need for exogenous TGF-β. Both *in vitro* and *in vivo* studies have reported that LIPUS induces the expression of type II collagen and PG in chondrocyte cells and cartilage tissue [215–220]. Choi *et al.* has shown that LIPUS of 200~300 mW/cm^2 increases the expression of type II collagen by 50% and PG by 30% in a 3-D alginate culture of human articular chondrocyte [221]. In the same culture, LIPUS treatment could reduce the matrix degradation by inhibiting the catabolic gene expression (e.g. MMP1) [214]. In the early stages of papain-induced arthritis in rats and full-thickness osteochondral defects in rabbits, LIPUS was shown to promote the repair process of arthritic cartilage [220]. In a rabbit OA model, LIPUS was reported to significantly reduce the severity of OA-induced

structural damage in the cartilage and synovium when combined with hyaluronate treatment [222].

Furthermore, LIPUS has been tested to efficiently induce the chondrogenic differentiation of MSCs both *in vitro* and *in vivo.* For example, LIPUS treatment of 200mW/cm^2 has been found to promote the chondrogenic marker expressions of COL2A1, ACAN, and Sox-9 in the early stages of rabbit MSC chondrogenesis [223]. The chondrogenesis of human MSCs seeded in a 3D scaffold was also enhanced when exposed to LIPUS [224]. In *in vivo* studies, LIPUS treatments greatly enhanced the chondrogenic differentiation of MSCs, reflected by increased total collagen and GAG content, compared to the control [225, 226]. LIPUS has also been reported to enhance the chondrogenesis process even without exogenous TGF-β, which is a well-known inducer of chondrogenesis [214]. The combined use of LIPUS and growth factor, TGF-β3, has been reported to improve the chondrogenic differentiation of a pellet culture of hMSCs [227]. In monolayer cultures, the synergistic use of ultrasound and TGF-β1 was observed to greatly enhance the expression of Sox9, aggrecan, and COL2A1 expression [228]. In addition, LIPUS was found to accelerate the proliferation of chondrocytes (harvested from rats and pigs) in monolayer cultures [229, 230].

Understanding of the effect of ultrasound on the biological behaviors of chondrocytes is still elusive. Many theories have been postulated. 1) The Calcium pathway; Even at low intensities, ultrasounds could increase the intracellular concentration of Ca^{2+} and that blocking this rise with intracellular calcium chelating agents, or inhibiting Ca^{2+}/ATPase, abolishes the stimulatory effect of ultrasound on PG synthesis [21]. 2) The MAPK/ERK pathway [96, 231]; The phosphorylation of FAK, Src, p130Cas, CrkII, and Erk1/2 may be increased in primary human chondrocytes under continuous LIPUS stimulation [231]. 3) The integrin/PI3K/Akt pathway; This pathway was found to be linked with the increased proliferation rate of primary pig articular chondrocytes in response to LIPUS [229].

3.5   Shock wave

An increasing number of studies have demonstrated the therapeutic effects of ESWT on the treatment or management of OA. ESWT has been shown to possess chondroprotective effects and cartilage repair roles [232–235], and to reduce the concentration of interleukin-10 and TNF-α in OA chondrocytes [236]. ESWT has also been shown to inhibit the expression of MMP1 and MMP3 in a rabbit OA model [237]. Furthermore, in this model, the chondroprotective effects of ESWT show time-dependence [235]. The application of 600 impulse shockwaves at 1.5×10^5 Pa each on the knee joint of OA rabbit models decreased the production of NO and rate of chondrocyte apoptosis [238]. However, excessive bouts of ESWT treatments or overdose of ESWT treatments may cause negative effects on OA cartilage repair, instead of inducing the favorable promotion [239, 105]. Studies have shown smaller denudations greater enhance chondrocyte formation and density for osteochondritis dissecans of rabbit knees when they are exposed to ESWT [240]. In addition, ESWT has also been reported to attenuate pain in human OA, enhancing the functional ability and decreasing the Western Ontario and McMaster University Osteoarthritis Index (WOMAC) pain score in the ESWT treatment group [241, 242]. ESWT

has also been shown to enhance the expression of crucial factors for chondrogenesis such as TGF-β, IGF, and fibroblast growth factors (FGFs) [243].

Although ESWT has shown positive effects of chondroprotection, subchondral bone remodeling, pain reduction, and motor function improvements, the exact mechanism remains unclear. The reduced expression of catabolic genes and inflammatory factors such as IL-1, IL-10, and TNF-α may partly contribute to the benefits of ESWT on OA patient. Greater investigation is required.

3.6 Other physical factors

Han *et al.* [170] found that Ca^{2+} signaling in chondrocytes occurs more quickly and with greater magnitude when the temperature is increased. This fact suggests that Ca^{2+} signaling of chondrocytes is also regulated by other, cellular, physical environments, such as ECM topography and temperature. Periodic heat shock at 41°C for 1 hour was found to significantly accelerate the chondrogenic differentiation of human mesenchymal stem cells in a pellet culture [244]. However, thermal stimulations of chondrogenesis have not been well explored. Studies also indicate that low energy light could directly stimulate chondrogenesis. LLLT using 660 and 780 nm wavelengths benefits the regeneration of OA by accelerating the initial breakdown of cartilage, destroyed by collagenase, and stimulating fibroblasts to synthesize the repairing collagen III [245]. UV irradiation is also normally used on engineered cartilage. A number of studies have shown the substrate's physical properties and its topological structures to be critical for the chondrocyte's differentiation, redifferentiation, and maturation [246]. A suitable substrate for seeding cells may help cartilage regeneration through mechanical, biological, and chemical effects in cartilage regeneration [247].

4 Conclusion

This review has elaborated on the application of physical stimulation, including electrical and electromagnetic field, mechanical force (tensile strain and fluid flow), ultrasound, shock wave, and others on bone and cartilage regeneration. A better understanding of how these factors act on the cells of bone and cartilage will allow better prediction and control in bone and cartilage tissue engineering approaches. In the review, the efficacy and mechanism of these biophysical stimulations were displayed. Pathway analyses among diverse classes of stimuli were described. The combination of different stimuli can result in synergistic therapeutic effects for the treatment of bone and cartilage diseases. Many pathways were postulated to explain the cell response to physical stimulation. Some pathways were complementary and overlapped, making it challenging to decipher the precise contribution and timing of activation of individual pathways. Yet, it is clear that the physical factors play very critical roles in the biological processes. The applications of tensile strain, shear stress, electromagnetic fields, and ultrasound are among many options to enhance osteogenesis and chondrogenesis of human stem cell. Therefore, direct physical intervention is an appealing approach and should be profoundly exploited to improve clinical outcomes in bone and cartilage regeneration.

However, when treating bone and cartilage disorders by physical intervention, both the positive and negative effects should be taken into consideration. There still exists the following major challenges in clinical use.

(1) The specific forms of physical stimulation and their dose effects and application timings must be carefully determined and validated. (2) Advanced techniques and devices must be addressed in achieving focalized stimulus delivery with adjustable signal type and intensity. Emphasis should especially be placed on how to monitor the healing process. The development of effective and reliable treatment protocols is a prerequisite. (3) Efficient *in vivo* assessing technologies must also be obtained. Although the positive effects of some physical factors have been identified *in vitro,* the exact value of these factors in native bone and cartilage tissues has not been determined. Welldesigned animal experiments need to be conducted as physical stimulation signal and dose effects on regeneration and repair of tissue should be firmly established and quantified. (4) An optimal strategy for bone and cartilage engineering may be to incorporate different physical stimuli with growth factors and biomaterials. How do the physical factors influence each other and what are the underlying mechanisms should be further investigated. (5) For cartilage repair, the low friction properties, collagen organization and crosslinking, as well as the related test standards should gain more emphasis.

Compared to bone, with its multiple cell types, vascularity, and high capability for innate repair, regeneration of cartilage faces more challenges [11]. Although the biochemical components of neocartilage (e.g. PG and collagen) can be controlled and modified, to date, no tissue-engineered neocartilage has been able to simultaneously match native cartilage's compressive, friction, and tensile properties under large deformations and motions [11, 192]. Joint forces over a large range of motion can take a devastating toll on neocartilage [11]. Therefore, the synthetic cartilage should have sufficient mechanical strength and compliance to sustain various forces and act as a cushion, respectively. In addition, the biomechanical response of native cartilage to loading varies widely and is time-dependent. Thus, a big challenge is defining, evaluating, and determining the specific mechanical properties, required for the replacement cartilages at specific implantation sites. Even if proper compressive properties match those of native tissues, the properties may not maintain when transplanted *in vivo.* Another challenge is how to properly integrate replacement tissues with the adjacent cartilage to provide stable, biologic fixation, load distribution, and proper mechanotransduction [248].

To some extent, within bone and cartilage cells, the signals from diverse stimuli overlap and the cells show a coordinated response. The combination of multiple stimuli may be better than any single stimulation to facilitate bone and cartilage regeneration. For example, enhanced differentiation of pre-osteoblasts was observed when simultaneously stimulating cells with cyclic strain and ultrasound [249]. To date, a combinatory and continuous application of strain, fluid shear, or electromagnetic fields with soluble small molecules is increasingly popular to supplement standard *in vitro* methods of inducing osteogenesis and chondrogenesis. Altogether, the success in repairing and regenerating musculoskeletal tissues depends on creating a musculoskeletal tissue with optimized mechanical, biological, and chemical characteristics. This requires interdisciplinary approaches and collaborative

work between biologists, physicists, medical doctors, and engineers to develop novel, effective regenerative therapies.

Acknowledgments

Abbreviation

GBR	guided bone regeneration
ACI	autologous chondrocyte implantation
hMSCs	human mesenchymal stem cells
ECM	extracellular matrix
ASCs	mouse adipose-derived mesenchymal stem cells
PFF	pulsating fluid flow
OPN	osteopontin
SSAT	spermidine/spermine N (1)- acetyltransferase
FAK	focal adhesion kinase (FAK)
RhoA	Ras homolog gene family member A
VSCC	voltage-sensitive calcium channels
ES	Electrical stimulation; EF: electric field
DC	direct current
CCEF	capacitive coupling electric field
EMF	electromagnetic field
AC	alternating current
PI3K	phosphatidylinositol-3-kinase
mTOR	mammalian target of rapamycin
TGF-β	transforming growth factor-β
$A_{2a}R$	adenosine A2A receptors
GAGs	glycosaminoglycans
PEMFs	pulsed electromagnetic fields
ELF-PEMF	extremely lowfrequency pulsed electromagnetic field
ROS	reactive oxygen species

Col I	collagen type I
GSK-3β	Glycogen synthase kinase-3 beta
TRK	tyrosine kinase receptor
TCF/LEF	T-cell factor/lymphoid enhancer factor
PI3K	phosphatidylinositide 3-kinases
TGF-β	transforming growth factor beta
BMP	bone morphogenetic proteins
AKT	protein kinase B
mTOR	mechanistic target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PGE2	Prostaglandin E2
AC	adenylyl cyclase
cAMP	cyclic adenosine monophosphate
PKA	protein kinase A
CREB	cAMP response element-binding protein
PKC	protein kinase C
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinases
FAK	focal adhesion kinase
GPCR	G protein-coupled receptor
OCN	osteocalcin
Osx	osterix
US	ultrasound
LIPUS	low intensity pulsed ultrasound
BSP	bone sialoprotein
MCP	monocytechemoattractant protein
MIP	macrophage-inflammatory protein
RANKL	receptor activator of nuclear factor kappa-B ligand
ATI	angiotensin II type I receptor

NO	nitric oxide
PGE2	prostaglandin E2
VEGF	vascular endothelial growth factor
GPCRs	G protein-coupled receptors
BMSCs	bone marrow-derived mesenchymal stem cells
MAPK	mitogen-activated protein kinase
ESWT	extracorporeal shock wave therapy
CBFA1	core-binding factor alpha l
ROCK	RhoA and Rho-associated protein kinase
PG	proteoglycan
ACAN	aggrecan
PRG4	proteoglycan 4
SZP	superficial zone protein
PCM	pericellular matrix
TRPV4	transient receptor potential vanilloid 4
CC	capacitive coupling
EPAC	exchange proteins activated directly by cyclic AMP
TNF-α	tumor necrosis factor α
NF-AT	nuclear factor of activated T-cells
SMFs	static magnetic fields
WOMAC	Western Ontario and McMaster University Osteoarthritis Index
LLLT	Low- level laser therapy

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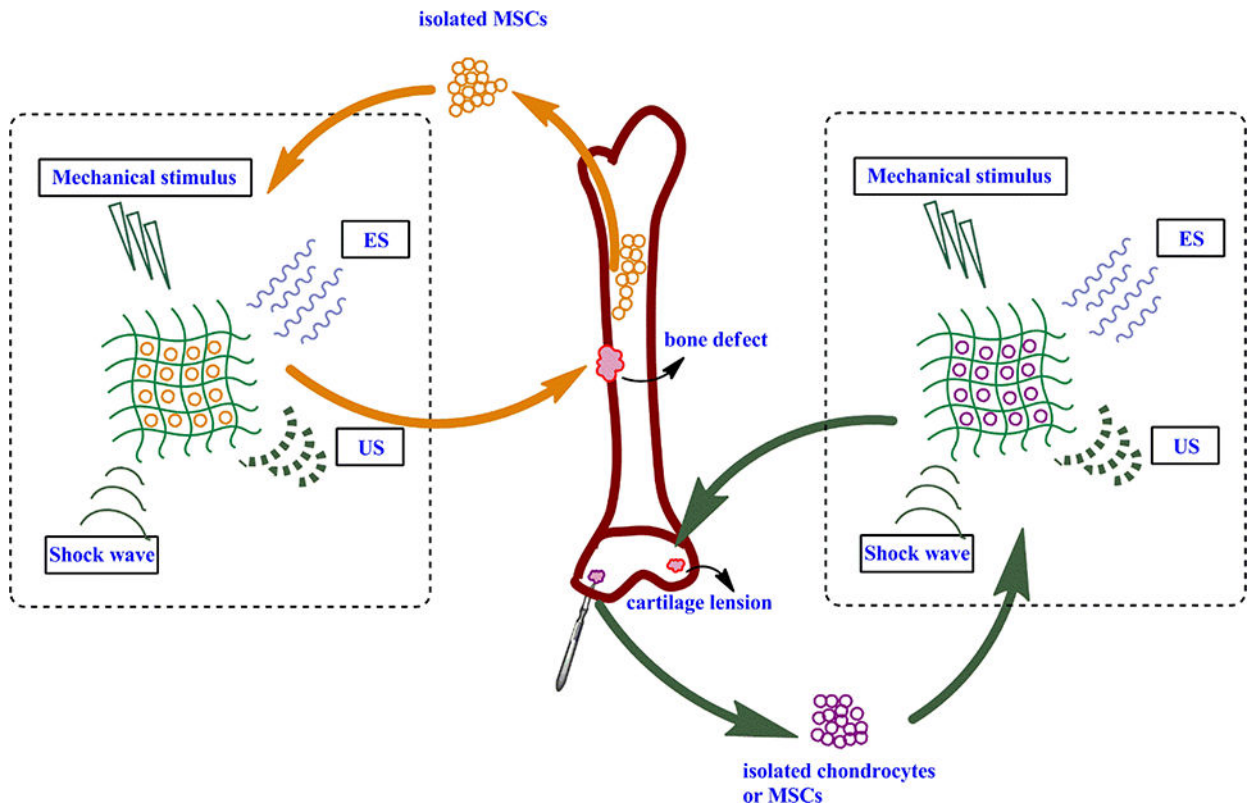
Fig. 1. Schematic of cell-based bone and cartilage regeneration from different physical stimulations.

ES, electrical stimulation; US, ultrasound; MSCs, mesenchymal stem cells.
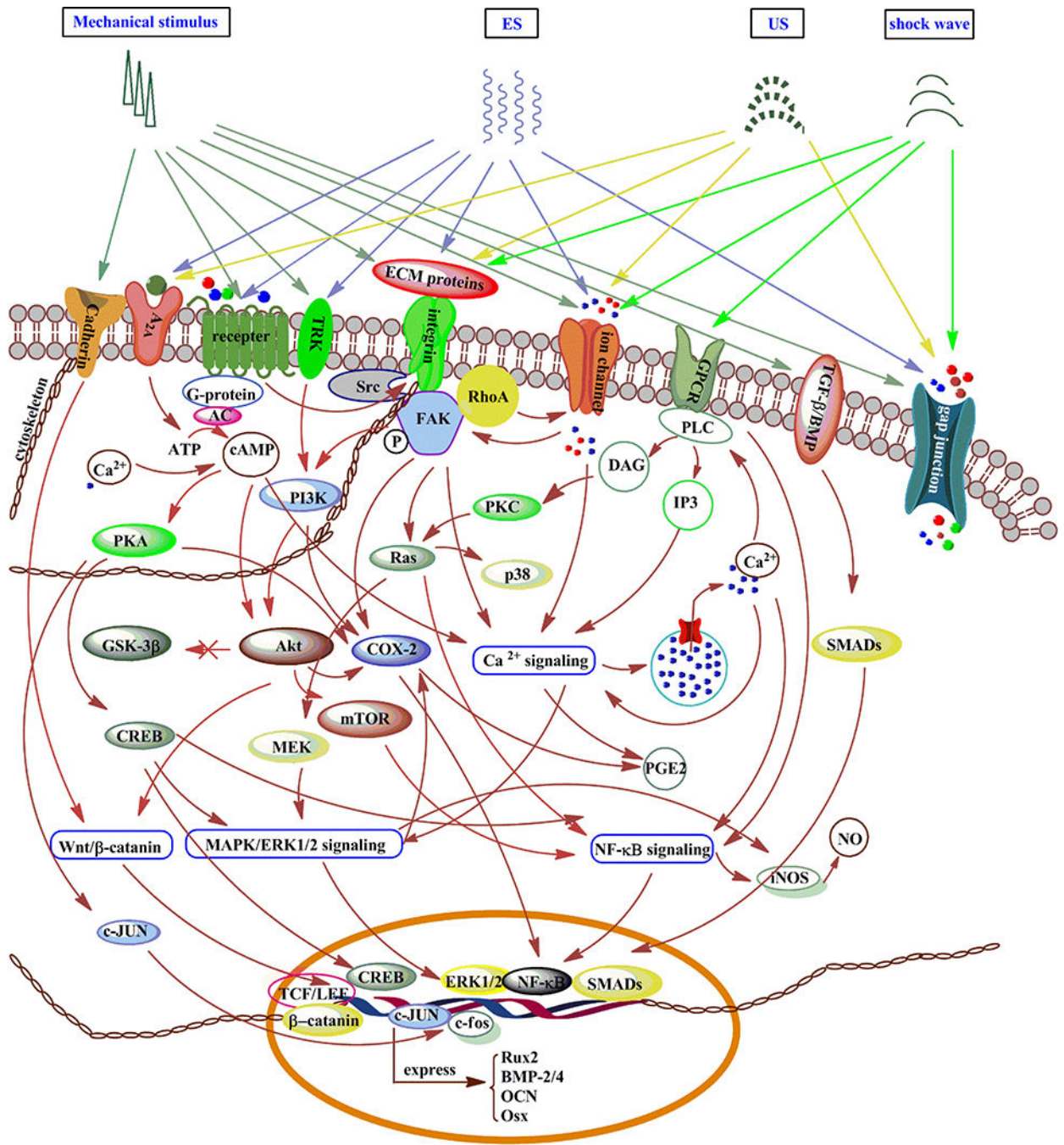
Fig. 2. Possible pathways involved in the biological response to mechanical stress, ES, US, and shock wave stimulations on bone cells.

Several pathways of MAPK/ERK, Wnt/β-catenin, PI3K/Akt, TGF-β/BMP, NF-κB, PKA, PKC, Ca^{2+} signaling could be regulated in response to biophysical stimulations, to enhance the cell proliferation and differentiation and to modulate the inflammatory response by modulating the expressing of bone markers Rux2, BMP2/4, OCM and Osx, etc. or other related regulators. GSK-3β (Glycogen synthase kinase-3 beta), TRK (tyrosine kinase receptor), TCF/LEF (T-cell factor/lymphoid enhancer factor), PI3K (phosphatidylinositide 3-kinases), TGF-β (transforming growth factor beta), BMP (bone morphogenetic proteins),

AKT (protein kinase B), mTOR (mechanistic target of rapamycin), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), PGE2 (Prostaglandin E2), AC (adenylyl cyclase), cAMP (cyclic adenosine monophosphate), PKA (protein kinase A), CREB (cAMP response element-binding protein), PKC (protein kinase C), MAPK (mitogen-activated protein kinase), ERK (extracellular signal-regulated kinases), FAK (focal adhesion kinase), GPCR (G protein-coupled receptor), OCN (osteocalcin), Osx (Osterix), ES (electrical stimulation), US(ultrasound), TRK (tyrosine kinase).
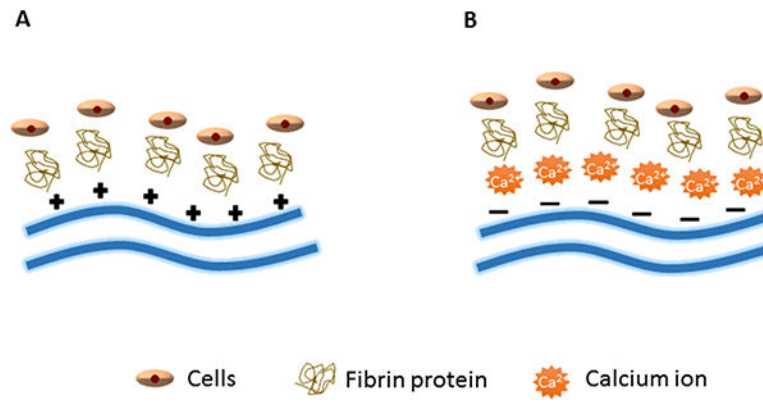
Fig. 3.
The biological mechanism of fibrin-mediated osteoconduction on (A) negatively and (B) positively charged surfaces.
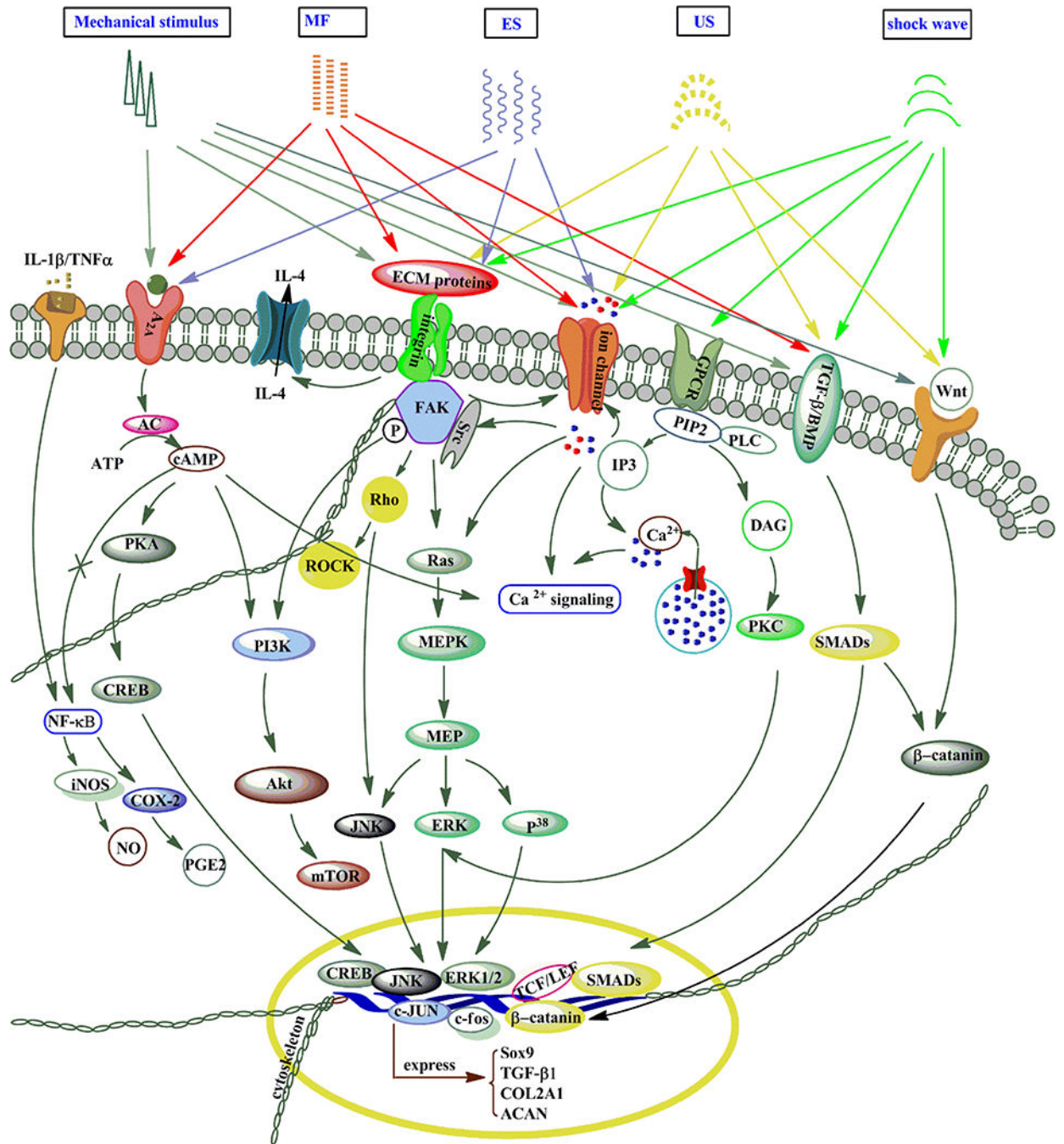
Fig. 4. Possible pathways are regulated in response to extra biophysical stimulations on chondrocytes.

Biophysical stimulations could act through one or a combination of MAPK/ERK, PKA/CREB, Wnt/β-catenin, PI3K/Akt, TGF-β/BMP, NF-κB, PKA, PKC, Ca^{2+} signaling pathways to enhance cell proliferation, survival, and differentiation and to modulate the inflammatory response by regulating the expressing of cartilage markers Sox9, TGF-β1, collagen type II, ACAN, etc.