RESEARCH ARTICLE

Physical stimulations and their osteogenesis-inducing mechanisms

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Abstract: Physical stimulations such as magnetic, electric and mechanical stimulation could enhance cell activity and promote bone formation in bone repair process *via* activating signal pathways, modulating ion channels, regulating bone-related gene expressions, *etc*. In this paper, bioeffects of physical stimulations on cell activity, tissue growth and bone healing were systematically summarized, which especially focused on their osteogenesis-inducing mechanisms. Detailedly, magnetic stimulation could produce Hall effect which improved the permeability of cell membrane and promoted the migration of ions, especially accelerating the extracellular calcium ions to pass through cell membrane. Electric stimulation could induce inverse piezoelectric effect which generated electric signals, accordingly up-regulating intracellular calcium levels and growth factor synthesis. And mechanical stimulation could produce mechanical signals which were converted into corresponding biochemical signals, thus activating various signaling pathways on cell membrane and inducing a series of gene expressions. Besides, bioeffects of physical stimulations combined with bone scaffolds which fabricated using 3D printing technology on bone cells were discussed. The equipments of physical stimulation system were described. The opportunities and challenges of physical stimulations were also presented from the perspective of bone repair.

Keywords: physical stimulations; cell activity; osteogenesis-inducing mechanisms; bone repair

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1. Introduction

Millions of people around the world are suffering from bone defects caused by bone diseases, traumas, infections, natural disasters, *etc*.[1,2] In USA alone, there are about seven million patients occurs bone defects[3]. In recent years, bone graft is widely used to treat bone defects, including autologous, allograft and artificial bone graft[4–7]. Artificial bone graft consists of seeding osteogenic cells onto 3D porous scaffolds which can be fabricated *via* 3D printing technology to induce osteogenesis. While there still exists delayed union or nonunion resulted from the loss of cell activity or cell death during bone defect repair. Chemical stimulations such as growth factors, osteogenic chemical inducers and hormones were utilized to improve the activity of bone cells[8–10]. Nonetheless, they displayed some disadvantages. For example, growth factors (such as bone morphogenetic proteins, transforming growth factors, *etc*.) display short half-life, and their activity is

lost rapidly[11]. Physical stimulations such as magnetic, electric and mechanical stimulation can constantly act on bone defect sites to enhance and maintain cell activity *via* activating signal pathways, modulating ion channels, regulating bone-related gene expressions, *etc*.[12] More importantly, physical stimulations have been proved to be safe and can control the bone growth direction depending on the direction of stimulations, thereby accelerating bone formation and regeneration[13–15].

Magnetic stimulation is a safe and non-invasive method to treat bone defect, which is produced by magnetic fields and electromagnetic fields, mainly including static magnetic field and pulse electromagnetic field stimulation[16–18]. Electric stimulation is a widely recognized approach for stimulating bone growth, which is produced by various currents, mainly including direct current, biphasic electric current and alternating electric current stimulation[19,20]. Mechanical stimulation is conducive to bone regeneration and healing, which is produced by ultrasound and other mechanical methods, mainly including ultrasonic, compressive stress, tensile stress and fluid shear stress stimulation[21,22]. Some summaries about the physical stimulations toward bone repair applications including classify and producing methods as well as advantages and disadvantages are presented in Table 1[16,23–32]. These physical stimulations can enhance cell activity and promote bone regeneration, which have been described as functional adaptation primarily owing to their osteogenesis-inducing ability. Detailedly, Hall effect produced by magnetic stimulations, inverse piezoelectric effect induced by electrical stimulations and mechanotransduction effect caused by mechanical stimulations can change local microenvironment of bone defect sites, alter cell membrane functions, activate signaling pathways, regulate bone-related gene expressions, *etc*., thereby enhancing cell activity and promoting bone regeneration[33–35]. These mechanisms are responsible for accelerating bone formation and bone repair.

In this paper, the different osteogenesis-inducing mechanisms of physical stimulations in bone repair process were systematically combed. The bioeffects of physical stimulations on cell behavior and bone formation which were investigated by numerous of studies *in vitro* and *in vivo* were summarized. Meanwhile, the synergetic effects of physical stimulations and bone scaffolds especially the 3D printed bone scaffolds on cells were presented. Besides, the equipment of physical stimulation systems were discussed, and the application prospects of these stimulations in bone repair were also analyzed.

2. The Osteogenesis-inducing Mechanisms of Physical Stimulations

Bone is a constantly updated tissue composed of metabolically active cells. Cell behaviors such as migration, proliferation, differentiation and apoptosis play a significant role in bone repair process. Physical stimulations can accelerate the proliferation and differentiation of osteoblasts and inhibit the formation of osteoclasts. In order to better understand the bioeffects of physical stimulations on cell activity and bone growth, the osteogenesis-inducing mechanisms of them are systematically combed according to relevant researches.

2.1 Magnetic Stimulation

The osteogenesis-inducing mechanisms of magnetic stimulation were explained as follows: (1) Producing Hall effect: The moving charged ions between bone matrix and osteocyte membrane would encounter a Lorentz force in magnetic field, and then form Hall voltage to induce the further migration of ions and

Table 1. Physical stimulations for bone repair.

Physical stimulations	Classify	Producing methods	Advantages	Disadvantages	References
Magnetic Stimulation	→Static magnetic field	→Magnets →Passing direct current through coils	→Safe →Non-invasive →No infection →No side effects →Ease of use	→Need for additional equipment →Less well defined stimulation site	[16,23,24]
	→Pulse electromagnetic field	→Passing pulse current through Helmholtz coils			
Electric Stimulation	→Biphasic current	→Biphasic current stimulator deliver biphasic stimulation currents	→Ease of operation →Stable strength →Reproducible	→The insufficient biocompatibility of electrodes can cause local infection	[25–28]
	→Direct current	→Passing direct electric current through electrodes			
	→Alternating current	→A generator produce alternating current			
Mechanical Stimulation	→Ultrasonic	→Ultrasound	→Safe →Non-invasive →No infection →Less complication	→Difficult to apply on freely moving animals →Difficult to precisely measure stimulus intensity	[29–32]
	→Compressive stress	→Compressive apparatus			
	→Tensile stress	→Tension apparatus			
	→Fluid shear stress	→Flow chamber			

improve permeability of cell membrane, thereby contributing the extracellular ions to pass through cell membrane to enhance cell activity[33]. (2) Improving cell membrane permeability: Phospholipid molecules on cell membrane possess diamagnetic anisotropy, they could be suffered magnetic field force, then rotated and orientated along the direction of the magnetic fields which caused the expansion of ion channels on cell membrane[36,37]. Therefore, numerous ions could pass through the cell membrane, and thus increasing conductivity and inducing much powerful current which produced a series of bioeffects to promote bone formation[38]. (3) Regulating calcium ions concentration: Calcium ions were the basic substance of all cells, which could affect the activity of intracellular enzymes, participate in cell signal transduction, regulate cell metabolism and cell activity, *etc*.[39,40] Magnetic fields could activate calcium ion-dependent protein kinase by altering calcium ions level, further regulate nuclear factors including cyclin which played a regulatory role in osteoblasts[41,42]. (4) Activating the cyclic adenosine monophosphate system: Magnetic stimulation could activate the cyclic adenosine monophosphate system, and then activate various enzyme systems which could induce bone cells to produce special physiological functions, thereby accelerating bone growth[43].

2.2 Electric Stimulation

The osteogenesis-inducing mechanisms of electric stimulation were explained as follows: (1) Inducing inverse piezoelectric effect: When an electric field was applied to bone defect sites, the stress and strain could be generated between the anode and cathode of the defect sites which could produce electric signals, thereby regulating bone cell behaviors[34]. (2) Up-regulating calcium level: Electric field could facilitate the calcium salt to move to the cathode[44], and elevate intracellular calcium level by promoting extracellular calcium ion influx into cells, thereby accelerating cells proliferation and bone tissue calcification and mineralization[14,45–49]. (3) Regulating growth factors: Electrical stimulation could regulate the expression of growth factors, such as insulin-like growth factors I and II, transforming growth factors, fibroblast growth factors, bone morphogenetic proteins, *etc*., thereby promoting bone formation[50–52]. (4) Changing local microenvironment: Electrical stimulation could improve local blood circulation and cause biochemical changes in the microcirculation around the bones and chondrocytes, such as elevated pH, thereby promoting ossification[53].

2.3 Mechanical Stimulation

The osteogenesis-inducing mechanisms of mechanical stimulation were explained as follows: (1) Mechanical stimulation could activate various signaling pathways when the stimulation acted on bone cells, and transduce the extracellular mechanical signals into the corresponding biochemical signals, such as Wnt receptors, integrins, insulin-like growth factor, G proteins and calcium ion channel[35], *etc*. (Figure 1), thereby inducing a series of gene expression to promote bone cell proliferation, differentiation, apoptosis[54,55]. (2) Mechanical stimulation could activate the calcium ion channel on cell membrane which could induce the extracellular calcium ion flow into the cell to increase the intracellular calcium concentration, thereby conducing to bone healing[56,57]. (3) The pressure wave produced by the ultrasound could enhance the fluid flow in the fracture area to increase the supply of nutrients and the removal of metabolites, and thus contributing to the proliferation and differentiation of osteoblasts and fibroblasts[58]. (4) Bone tissues possess abundant interconnected microchannels, mechanical stress could produce strain gradients and cause ionic current flow along the microchannels, which played an important role in the process of mechanotransduction[59].

3. The Effects of Physical Stimulations on Bone Cells

The effectiveness of physical stimulations in bone repair has been investigated *in vitro* and *in vivo*. It was proved that physical stimulations could promote bone mesenchymal stem cells differentiate to osteoblasts, accelerate osteoblasts proliferation and differentiation, and inhibit osteoclasts formation, thereby contributing to bone repair and regeneration.

3.1 Magnetic Stimulation on Bone Cells

The studies of magnetic stimulation used to stimulate bone cells were mainly focused on the static magnetic field and pulse electromagnetic field. In general, static magnetic field could promote osteoblasts proliferation and differentiation as well as inhibited osteoclasts formation, thereby promoting the process of bone repair[60]. Moreover, the strong static magnetic field (> 1 T) could regulate the orientation of bone cells and matrix proteins. Yamamoto *et al*.[61] investigated the effects of 0.16 T static magnetic field continuously exposed 20 days on the rat calvaria cell and found that static magnetic field significantly increased activity of alkaline phosphatase and osteocalcin content. Zhang *et al*.[62,63] investigated the bioeffects of 16 T static magnetic field on osteoblasts and osteoclasts. They found that static magnetic field enhanced osteoblast differentiation determined by the formed nodules area and the calcium deposition, and inhibited osteoclast formation evaluated by tartrate-resistant acid phosphatase, integrin β3, matrix metalloproteinase 9, receptor activator of nuclear

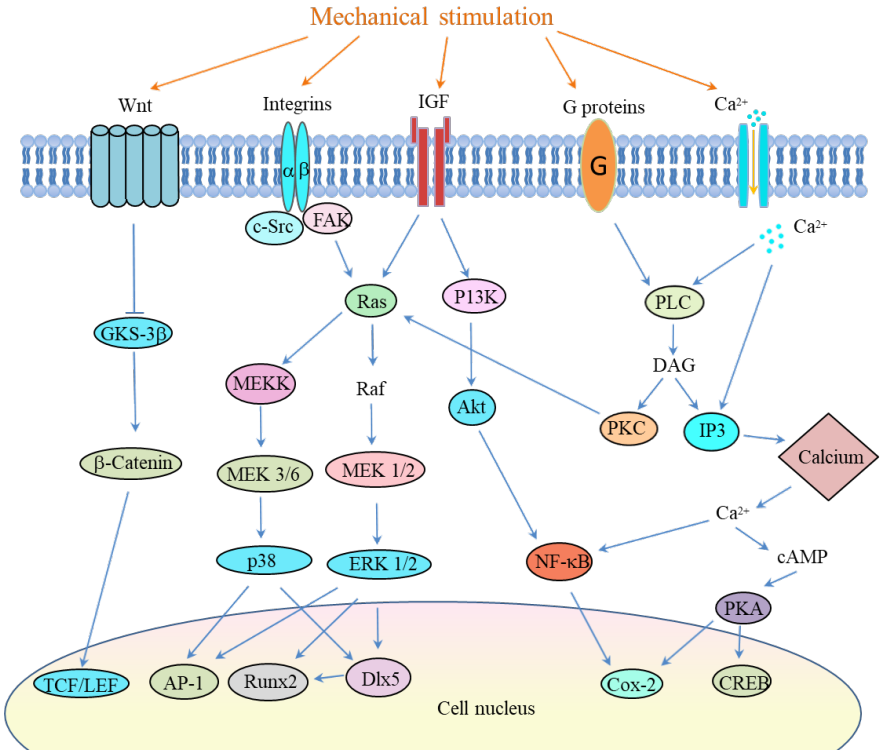

Figure 1. Schematic of interactions of various signaling pathways under mechanical stimulation. Wnt receptors, integrins, insulin-like growth factor (IGF), G proteins (G) and Ca^{2+} channels were stimulated by mechanical stimulation, thereby inducing a series of transcription factors to regulate osteoblast proliferation and differentiation.

factor κB ligand, *etc*. Di *et al*.[64] also found that 16 T static magnetic field inhibited osteoclasts formation and differentiation due to the decreases of tartrate-resistant acid phosphatase activity, and resulted in osteoclasts apoptosis and necrosis. Kotani *et al*.[36] found that 8 T static magnetic field stimulated the osteoblast transformed to rodlike shapes, cells differentiation and matrix synthesis. Moreover, static magnetic field regulated the orientation of cells and bone formation parallel to the static magnetic field direction (Figure 2B and 2C). Some studies have shown that magnetic nanoparticles in 3D printed scaffolds could also produce magnetic stimulation on bone cells[65–67]. Huang *et al*. investigated the effects of magnetic stimulation which produced by incorporation of Fe_2O_3 magnetic nanoparticle in polylactic-co-glycolic acid/collagen/hydroxyapatite composite scaffolds on bone mesenchymal stem cells. They found that the magnetic nanocomposite scaffolds obviously enhanced the proliferation and differentiation of bone mesenchymal stem cells[65].

Pulse electromagnetic field could induce bone formation as well as inhibit bone resorption by regulating the osteoblasts and osteoclasts formation, proliferation and differentiation. The effectiveness of regulation depended on the magnetic field intensity. It was owing to the nonlinear intensity window effect of pulse electromagnetic field in the process of regulating cell behaviors[68]. Zhou *et al*.[69] investigated the bioeffects of 50 Hz sinusoidal electromagnetic fields at different intensities (0.9 mT, 1.2 mT, 1.5 mT, 1.8 mT, 2.1 mT, 2.4 mT, 2.7 mT and 3.0 mT) on the osteoblasts differentiation and Collagen-I mRNA and bone morphogenetic protein-2 expression. The results showed that the electromagnetic fields at 1.5 ~ 2.4 mT groups significantly increased the osteoblasts differentiation and the expression of Collagen-I mRNA and bone morphogenetic protein-2. Moreover, the calcium content and calcified nodules of the 1.8 mT group were highest than other groups. Kamolmatyakul *et al*.[70] reported that pulse electromagnetic field (50 Hz, 1.5 mV/cm) significantly increased the proliferation rate of osteoblast-like cells. Diniz *et al*.[71] proposed that pulse electromagnetic field (15 Hz, 7 mT) could promote osteoblasts differentiation in the proliferation and differentiation stage, and they pointed out that the promotion was not associated with the increased number of cells. Wang *et al*.[72] investigated the effects of 15 Hz pulse electromagnetic field with various intensities of 0, 0.5, 1, 2 and 3 mT on osteoclast. The results showed that 0.5 mT pulse electromagnetic field significantly inhibited the osteoclast formation and maturation. Chang

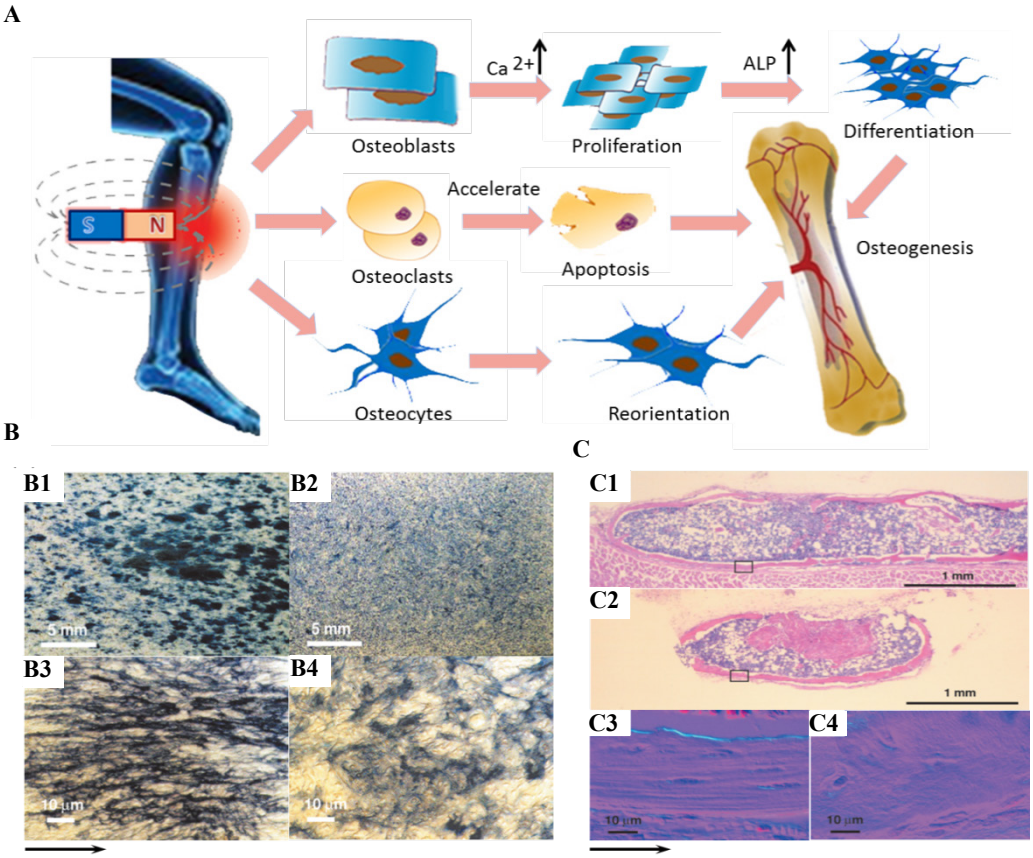

Figure 2. (**A**) Schematic of static magnetic field promote osteogenesis. (**B**) Effects of the static magnetic field on the cell differentiation: the alkaline phosphatase activity was increased in exposed groups (**B1**, **B3**) compared with control groups (**B2**, **B4**). The orientation of cells was maintained parallel to the direction of static magnetic field. (**C**) Effects of the static magnetic field on the bone formation: the bone formation in exposed groups (**C1**) were significantly increased compared with control groups (**C2**). The orientation of bone formation was parallel to the direction of static magnetic field. The squares in (**C1**) and (**C2**) represent the areas in (**C3**) and (**C4**), respectively. The arrow indicated the direction of static magnetic field[36].

et al.[73] examined the effects of pulse electromagnetic field (7.5Hz, 4.8 V/cm) on osteoclasts, and they found that the pulse electromagnetic field obviously inhibited the osteoclastogenesis.

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3.2 Electric Stimulation on Bone Cells

The main sources of electric stimulation on bone cells are biphasic electric current, direct current and alternating electric current. The action modes and intensity of electric current have a significant influence on cell behaviors. Kim *et al*.[14] investigated the effect of biphasic electric current (1.5 μA/cm2, 3000 Hz) on the proliferation, differentiation and synthesize cytokines of osteoblasts in the interrupted and continuous modes. The results showed that the proliferation of osteoblasts increased 31% after continuous stimulate 2 days whereas unchanged in the interrupted mode, indicating that the continuous stimulation was more effective than interrupted stimulation. The bone mesenchymal stem cells possess the capability to osteogenic differentiation which could effectively accelerate bone healing and bone remodeling, so the migration of bone mesenchymal stem cells play an important role in bone repair. Electric field could promote the migration of bone mesenchymal stem cells, and the migration rate of the bone mesenchymal

stem cells was controlled by the electric field intensity[74]. Banks *et al*.[75] were also verified this viewpoint, and found that the bone mesenchymal stem cells became significantly elongated and were perpendicular to the electric field vector. Creecy *et al*.[76] exposed bone mesenchymal stem cells to either 10 or 40 mA alternating electric current for 6 h/day, and they found that the stimulations significantly increased the gene expressions of osteopontin, osteocalcin and runt-related transcription factor 2, thereby promoting the differentiation of bone mesenchymal stem cells to osteoblasts. Wang *et al*.[77] reported that direct current electric stimulation promoted bone mesenchymal stem cells migration. The optimal intensity and duration were 200 mV/mm and 4 h, respectively, and they up-regulated the osteocalcin, alkaline phosphatase and runt-related transcription factor 2 expressions which benefited to bone mesenchymal stem cells proliferation and differentiation. In addition, some scholars investigated the cell responses to electrical stimulation which combined with 3D printed bone scaffolds[78,79]. Grunert *et al*. studied the effects of electric stimulation on osteoblasts which cultured on 3D printed calcium phosphate/collagen composite scaffolds. The results indicated that the stimulation promoted the proliferation and differentiation of osteoblasts[78].

3.3 Mechanical Stimulation on Bone Cells

The mechanical stimulation is mainly including the ultrasonic stimulation and mechanical stress stimulation. The ultrasound is a high frequency mechanical wave which can be transmitted into biological tissues to produce biochemical reactions[80]. Mechanical stresses are mainly divided into compressive stress, tensile stress and fluid shear stress. The effects of mechanical stimulation on bone cells mainly depend on loading mode, intensity, frequency and duration. The low intensity pulsed ultrasound (< 100 mW/cm^2) could modulate the proliferation and differentiation of osteoblasts and osteoclast through regulating bone-related gene expressions, and the regulatory effects were related with intensity[21,81,82]. Yang *et al*.[83] examined the effect of ultrasonic stimulation with different intensities (62.5 mW/cm^2, 125 mW/cm^2 and 250 mW/cm^2) on the osteoblasts differentiation and osteoclastogenesis. The results indicated that the 125 mW/cm^2 ultrasound at obviously enhanced the mineralization, collagen synthesis and alkaline phosphatase activity of osteoblasts. Moreover, low intensity pulsed ultrasound at 62.5 and 125 mW/cm^2 significantly inhibited the formation and differentiation of osteoclasts. Sun *et al*.[84] reported that low intensity pulsed ultrasound (1 MHz, 68 mW/cm^2) obvious increased the osteoblast cell counts and alkaline phosphatase level after ultrasonic stimulation for 7 days, and significantly reduced the osteoclast cell counts. Korstjens *et al*.[85] found that low intensity pulsed ultrasound (1.5 MHz, 30 mW/cm^2) treated at 20 min/day for 3 or 6 days significantly increased the bone collar volume and calcified cartilage. It was worth noting that ultrasound stimulation displayed pronounced biological effects on cells which cultured on 3D printed bone scaffolds[62,86,87]. Zhou *et al*. investigated the effects of low intensity pulsed ultrasound on human bone marrow mesenchymal stem cells seeded on hydroxyapatite scaffolds, and they found that the ultrasound stimulation combined with scaffolds significantly improved the alkaline phosphatase activity and calcium deposition[86].

The mechanical stresses with various peak stress amplitude, frequency and duration have different influences on cell behaviors. Bone cells could distinguish different stress magnitude and adjust the bio-chemical response accordingly. Tang *et al*.[88] investigated the bioeffects of cyclic stretching (500 , 1000 and 1500) on osteoblasts. The results indicated that the stretching at 500 increased osteoblast collagen synthesis, while the stretching at 1000 and 1500 inhibited collagen production, indicating that the response of osteoblasts was dependent on the stretching magnitude. Jagodzinski *et al*.[89] proved the mechanical strain with an elongation of 2% and 8% increased the alkaline phosphatase levels and osteocalcin secretion of mesenchymal stem cells after loading 4 days, and the increased rate of 8% stretching group was higher than 2% stretching group. Kearney *et al*.[90] found that the 2.5%, 0.17 Hz cyclic tensile mechanical strain obviously reduced mesenchymal stem cells proliferation after 2 and 3days, and increased the expression of transcription factor Cbfa1, osteocalcin, collagen type I and bone morphogenetic protein-2 which related to osteogenic differentiation (Figure 3A). Sanchez *et al*.[91] reported the cyclic compression stress (1 MPa at 1 Hz) significantly increased the genes expression of cyclooxygenase 2, interleukin-6, receptor activator of nuclear factor κB ligand, *etc*. which involved in bone remodeling and bone formation. Li *et al*.[92] investigated the bioeffects of different oscillating frequencies, peak shear stress amplitudes, and total flow durations on osteocyte activity. The results indicated that the three dynamic fluid flow parameters could regulate the osteocyte activity, and faster oscillating frequencies, higher peak shear stress amplitudes and longer loading durations were beneficial to bone formation. Liu *et al*.[93] proved that fluid shear stress at 1.6 and 1.9 Pa significantly induced the cell elongation and reorientation parallel to the direction of fluid flow, indicating that the fluid shear stress could influence the cell growth direction. Li *et al*.[94] found that the fluid shear stress at 12 dyn/cm^2 could reorganize the cytoskeleton in MC3T3-E1 pre-

osteoblasts which was critical for mechanosensation and intracellular signal transduction. And the actin filaments rapidly reorganized into thick parallel bundles of fibres, and the fibre formation was induced by shear stress loading 0-90 min whereas the cytoskeleton was disrupted over loading 90 min (Figure 3B). Besides, fluid shear stress could produce bioeffects to cells which seeded on 3D printed bone scaffolds[95,96]. Stiehler *et al*. studied the effect of fluid shear stress on human mesenchymal stem cells cultured on porous poly(D,L-lactide-co-glycolide) scaffolds, and the results showed that the fluid shear stress markedly enhanced alkaline phosphatase activity, increased Ca^{2+} content and promoted cells growth[95].

3.4 Physical Stimulations on Artificial Bone

In terms of bone defects repair, bone scaffolds need to possess interconnected internal porous structures that provide channels for the adhesion and migration of bone cells, the transmission of nutrients, and the growth of bone tissue[97]. Meanwhile, bone scaffolds also need to possess customized external geometries that can exactly match bone defects, which is beneficial for the structural and functional remodeling of bone[98]. The customized porous scaffolds present a great challenge for manufacturing process. 3D printing is one of the advanced manufacturing technologies which fabricate objects directly from the given computer-aided design model *via* layer by layer printing. It can fabricate the interconnected internal porous structure and the customized external shape of bone scaffolds. Moreover, bone scaffolds require excellent biocompatibility to encourage cell adhesion and migration[99]. Bioceramics (such as hydroxyapatite, bioactive glass, *etc*.) and biopolymers (such as polycaprolactone, polylactide, *etc*.) are suitable materials for the fabrication of bone scaffolds owing to their good biological properties[100–102]. Magnetic materials (such as Fe_3O_4, γ-Fe_2O_3, *etc*.) and conductive materials (such as carbon nanotube, graphene, *etc*.) are incorporated in bioceramics and/or biopolymers to enhance the biological and physical properties of scaffolds[103–106]. Zhang *et al* incorporated Fe_3O_4 nanoparticles into polycaprolactone and mesoporous bioactive glass composites, and found that the 3D printed composite scaffold significantly stimulated cells proliferation and differentiation[107]. Therefore, the bone scaffold fabricated *via* 3D printing technology with

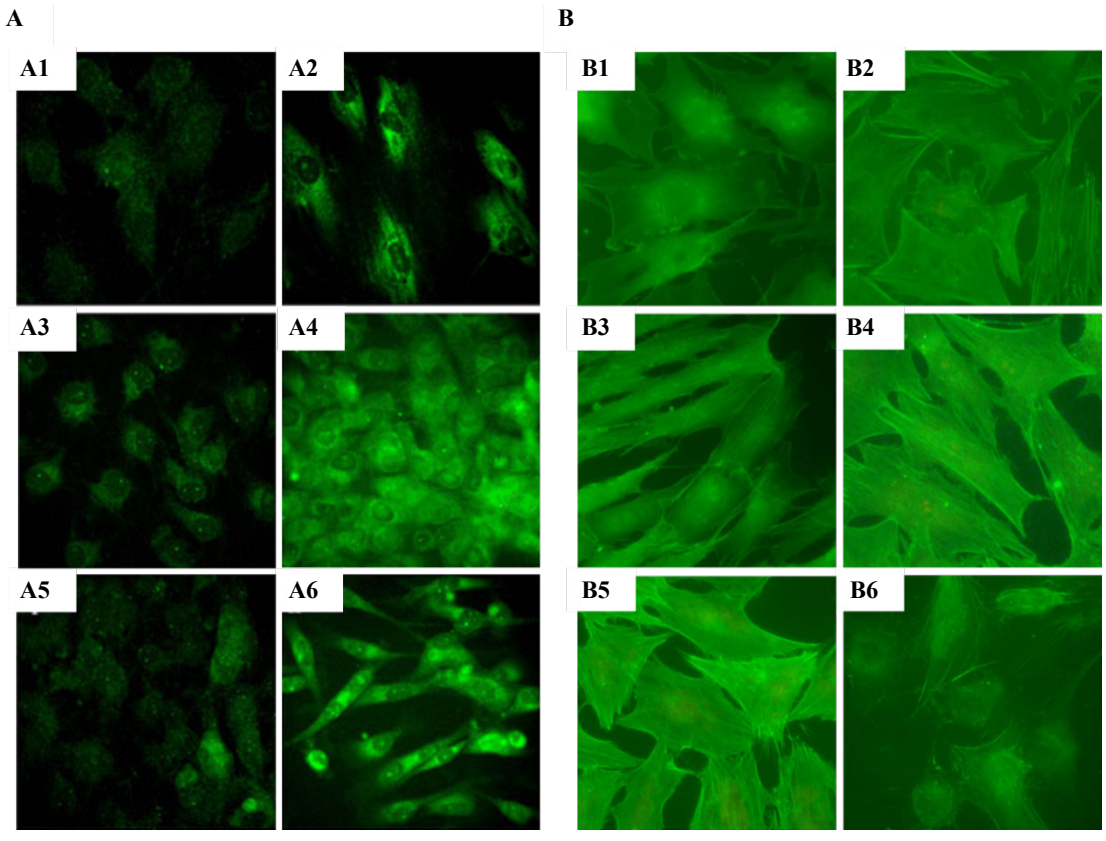

Figure 3. (**A**) Strain induced the expression of the osteogenic markers Cbf1 (**A1**, **A2**), collagen type I (**A3**, **A4**), and osteocalcin (**A5**, **A6**). (**A1**, **A3**, **A5**) mesenchymal stem cells in static culture for 6 days, (**A2**, **A4**, **A6**) mesenchymal stem cells exposed to mechanical strain (2.5%) after 6 days[90]. (**B**) The fluid shear stress at 12 dyn/cm^2 induced stress fibre formation in different time spans. (**B1 - B6**) The cells were loaded for 0, 5, 15, 45, 90 and 120 min, respectively)[94].

physical stimulations (such as magnetic or conductive materials) is a promising and efficient candidate for bone formation and healing.

The physical stimulations combined with bone scaffolds has great potential in bone repair because they can fully reflect the synergetic effects of bone scaffolds and physical fields in bone repair process. Yun *et al*.[66] found that static magnetic field synergized with magnetic bone scaffolds promoted the osteoblastic differentiation including enhanced alkaline phosphatase activity and up-regulated gene expressions of osterix and runt-related transcription factor 2. Feng *et al*.[67] investigated the bioeffects of 4000 G static magnetic field on the osteoblasts cultured on poly-L-lactide substrates surface and found that alkaline phosphatase activity was significantly increased, indicating that static magnetic field combined with scaffolds could promote cell differentiation. Arjmand *et al*.[108] proved that the extremely low frequency pulse electromagnetic field combined with polycaprolactone (PCL) nanofibrous scaffold significantly enhanced the proliferation and osteogenic differentiation of mesenchymal stem cells by analyzing alizarin red staining, alkaline phosphatase activity, calcium content, related genes expressions such as collagen type I, runt-related gene 2, osteonectin and osteocalcin. Some studies have shown that scaffold materials have a significant impact on bone repair[109,110]. Jin *et al*.[79] investigated the effects of electric stimulation combined with three-dimensional porous scaffolds (PCL, PCL/carbon nanotubes (CNT) and PCL/ -tricalcium phosphate (-TCP) scaffold) on the osteoblasts. They found that the electric stimulation enhanced the alkaline phosphatase activity and calcium mineralization of osteoblasts in all scaffolds, and the enhancement of bone mineralization in PCL/-TCP scaffold was the highest (Figure 4A). The results indicated that the electric stimulation and scaffold materials both played a significant role in bone repair. Sun *et al*.[111] reported that the electric stimulation induced the reorientation of fibroblasts in three-dimensional collagen scaffold and along the direction of the electric stimulation. Chen *et al*.[96] investigated synergistic action of fluid shear stress and three-dimensional porous scaffolds (collagen/hydroxyapatite, Col/HA) on the biological behaviors of mesenchymal stem cells. The results showed that the viability of mesenchymal stem cells in the all scaffolds was significantly increased under oscillatory shear stress cultured for 3 weeks compared with control group. Moreover, the oscillatory shear stress significantly enhanced the osteogenic differentiation of mesenchymal stem cells in the scaffolds (Figure 4B).

4. *In vivo* Studies of Physical Stimulation

In vivo studies mainly include animal experiments and clinical trials. Animal experiments can provide theoretical supports for clinical trials. Many animal experiments and clinical trials have been carried out to determine the effects of magnetic, electric and mechanical stimulation on bone repair[112–115].

4.1 *In vivo* Studies of Magnetic Stimulation

The magnetic stimulation produced by magnetic fields and electromagnetic fields could conduce to accelerate bone repair due to that they could promote bone formation and inhibit bone resorption[116–119]. Taniguchi *et*

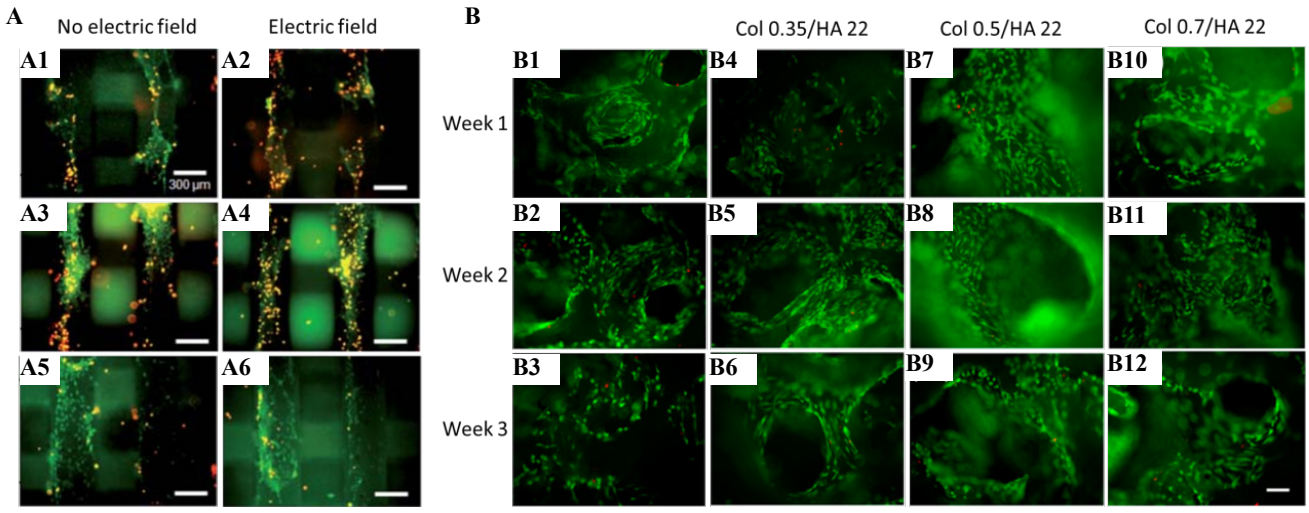

Figure 4. (**A**) Live/dead assay of MG63 cells seeded on PCL (**A1**, **A2**), PCL/CNT (**A3**, **A4**), and PCL/-TCP scaffolds (**A5**, **A6**) with and without electric stimulation after 14 days[79]. (**B**) Live/dead assay of mesenchymal stem cells seeded on the midline section of different scaffolds for 1, 2, and 3 weeks under oscillatory perfusion. (**B1**-**B3**) Static culture mesenchymal stem cells. The scale bar indicates 50 μm. Living cells (green) and dead cells (red)[96].

al.[120,121] investigated the effects of 30 mT static magnetic field on the bone mineral density of ovariectomized rat model under whole body exposure. The results indicated that bone mineral density was significantly increased after 12 weeks because static magnetic field increased the level of locomotor activity in rats model. Besides, the bone mass was also higher than control group. Puricelli *et al*.[122,123] assessed the effect of the magnetized metal device on the femur cavity of rats. The results showed that the 4.1 mT magnetic field accelerated the bone formation compared with control group on days 15, 45, 60 after the implantation, thereby enhancing the bone healing. Leesungbok *et al*.[124] compared the bone formation ability of titanium implant with or without magnets in a rabbit tibia. The results showed that the titanium implants with magnet enhanced early implant bone formation compared with without magnet implants. It was worth noting that static magnetic field combined with magnetic nanocomposite scaffolds consisting of polymer and magnetic nanoparticles could combine the advantages of them in bone repair. Yun *et al*.[66] investigated the effects of static magnetic field synergized with magnetic scaffolds on osteoblastic functions of mouse calvarium. The results showed that static magnetic field combined with magnetic scaffolds significantly enhanced the new bone formation after exposure 6 weeks (Figure 5). Inoue *et al*.[125] investigated the effects of pulse electromagnetic field on late bone healing phase of canine mid-tibia osteotomy model. The results revealed that the stimulation of 1h/day for 8 weeks significantly increased the new bone formation and mechanical strength. Zaki *et al*.[126] researched the effectiveness of pulse electromagnetic field on the fractures healing of patients at different treatment stages. The results showed that pulse electromagnetic field significantly accelerated the bone healing of patients at 12 weeks who were continued subjecting to pulse electromagnetic field treatment compared with control group which only treated with plaster cast, and the late treatment group which received pulse electromagnetic field treatment after the cast was removed also increased the osteocalcin level, indicating that the pulse electromagnetic field could enhance the fracture healing.

4.2 *In vivo* Studies of Electric Stimulation

Bone tissue would respond to electrical stimulation signals which produced by various electric currents, thereby generating a series of biochemical reactions which were conducive to bone repair. El-Hakim *et al*.[127] investigated the effects of direct current of 10 A on mandibular distraction osteogenesis of adult goats in different distraction periods. The results showed that direct current played a positive role on mandibular distraction osteogenesis when it was applied to the distraction areas during activation and consolidation periods. Fredericks *et al*.[128] reported that the direct

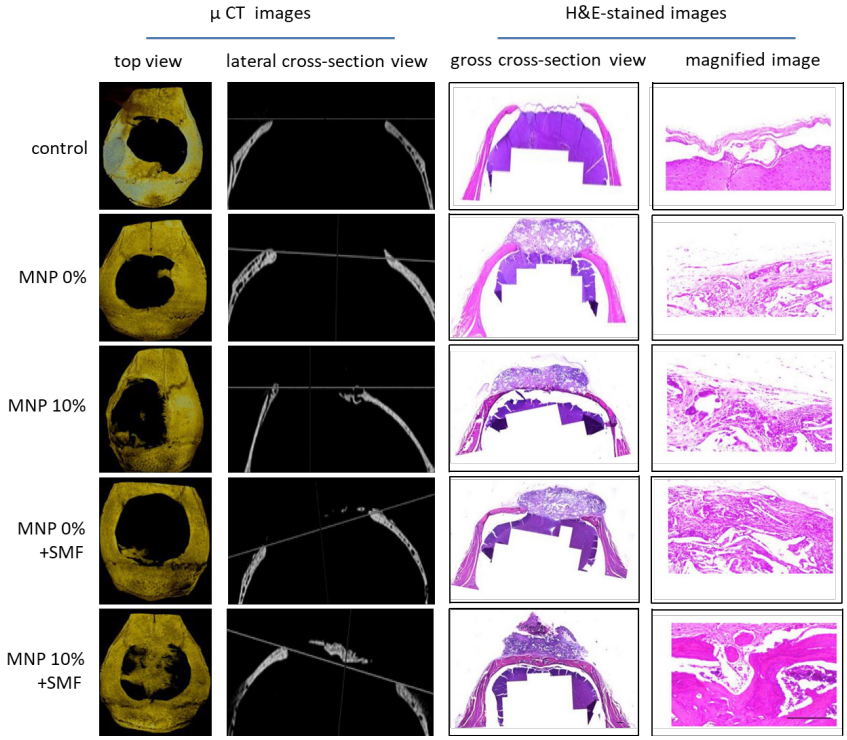

Figure 5. Effects of static magnetic field and PCL/MNP (magnetic nanoparticle) scaffolds on bone regeneration of calvarial defect in mouse after 6 weeks of implantation[66].

current stimulation could promote bone formation of rabbit posterolateral fusion model. Park *et al*.[129] investigated the effects of electric stimulation of 1h/day for 4 weeks on 3 mm gapped osteotomies in mid-tibial of rabbit models, the two electrodes were placed on the above patellar tendon and lateral thigh, respectively. The results showed that the callus area and mineral content were 27% and 31% higher than control osteotomies, respectively, and the biomechanical properties were significantly higher than control group. It indicated that the electric stimulation could increase the mineralization and callus development of the bone healing regions, thereby enhancing the biomechanical properties. Chen *et al*.[130] evaluated the changes in bone mineral density of fifteen males with spinal cord injury after the intervention of functional electric stimulation 6 months. The results showed that the bone mineral density was increased significantly, whereas the effect would disappear when the stimulation was removed.

4.3 In vivo Studies of Mechanical Stimulation

Mechanical stimulation can accelerate the bone repair process and induce healing of nonunions, which depends on the intensity, frequency and duration of loading. Azuma *et al*.[131] investigated the effects of ultrasonic stimulation (30 mW/cm^2, 20 min/day) on fracture healing in the different duration (days 1–8, 9–16, 17–24 and 1–24). The radiography and histological results demonstrated that the low intensity pulsed ultrasound could accelerate fracture healing at each treatment period, and the 1–24 days group was more effective than other treated groups (Figure 6). Moreover, the mechanical torsion properties of treated femurs were significantly higher than nontreated femurs, and the properties in the 1–24 days group were the highest. Takikawa *et al*.[132] established nonunion model of tibia fracture in rat, and utilized low intensity pulsed ultrasound (30 mW/cm^2) to treat the fracture sites. They found that the healing rates of tibia samples were 30.8% and 50% after treated for 4 weeks and 6 weeks, respectively, while the samples in control group were not healing. Nolte *et al*.[115] studied the bioeffect of low intensity pulsed ultrasound (20 min/day) on the fracture nonunion sites of 29 patients. The results showed that 86% of patients obtained complete healing after 22 weeks. Fritton *et al*.[133] investigated the skeletal responsed to compressive loads by applying controlled cyclic axial load on mouse tibia and analyzed the bone mineral content of loaded and unloaded

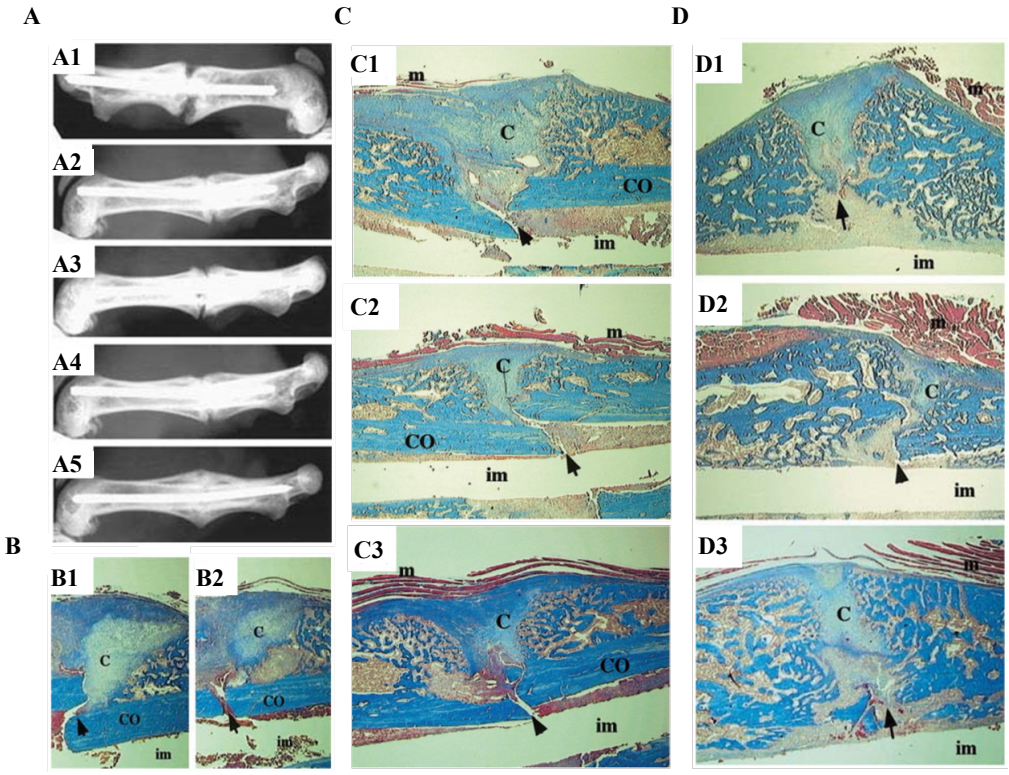

Figure 6. Effects of low intensity pulsed ultrasound on fracture healing in the different duration. (**A**) Radiography of treated femur (**A2-A5**, treatment duration at days 1–8, 9–16, 17–24 and 1–24, respectively) and nontreated femur (**A1**) at day 25 after fracture. The treated groups had better bone healing than control group. (**B–D**) Histological analyses of low intensity pulsed ultrasound treatment on fracture healing at different duration. At day 9, early endochondral ossification in treated femur (**B2**) was greater than in the control (**B1**). At day 17, endochondral ossification and remodeling in the control femur (**C1**) were less than treated femur 16 days (days 1–16, **C2**) and 8 days (days 9–16, **C3**). At day 25, bone bridging in the control femur (**D1**) was less than treated 24 days (days 1–24, **D2**) and 8 days (days 17–24, **D3**)[131].

limbs. The results showed that the average trabecular thickness, bone mineral content and bone volume fraction increased 12%, 14% and 15%, respectively. Lambers *et al*.[134] studied the effects of cyclically load of 8 N on the bone formation and resorption of mouse tail vertebrae. The results showed that the 8 N group significantly increased trabecular bone volume fraction and cortical area fraction. Moreover, the bone strength increased due to the increasing of bone formation area and the decreasing of bone resorption area (Figure 7). Peptan *et al*.[135] investigated the effects of cyclic tensile or compressive forces (1 N, 8 Hz) on remodeling and growth of intramembranous bone and cranial sutures of rabbit models. The results showed that the high-frequency cyclic tensile and compressive forces both induced the modeling and growth of cranial sutures.

5. The Equipments of Physical Stimulation Systems

In vitro and *in vivo* studies had shown that different physical stimulations had different effects on bone cells. As the source of physical stimulations, the physical stimulation systems play an important role in bone repair. To date, there are no unified stimulation systems for each kind of physical stimulation, and the representative physical stimulation systems are shown in Figure 8.

5.1 The Magnetic Stimulation Systems

The static magnetic field and pulse electromagnetic field were widely used in the treatment of various bone-related diseases such as fracture, osteoporosis, bone delayed union or nonunion, *etc*., owing to their non-invasive, no infection, no side effects and ease of use[120,126,138]. *In vivo* studies, many researchers implanted magnet rods, magnetic plates and magnetic washers into bone defect sites to construct static magnetic field[118,121-124,139,140]. Some researchers constructed static magnetic field stimulation equipment composed of magnetic plates which fixed on outside of cage (Figure 8A)[120] or utilized signal generator to produce direct current which transferred to a pair of Helmholtz coils to expose animals[141]. *In vitro* studies, the static magnetic field exposure systems which used to expose bone cells had various modes, such as magnets, a magnetic shield box, parallel arranged magnetic plates, *etc*.[36,142–146]. The construction of pulse electromagnetic field usually adopted the tunable pulse generator to produce pulse current with specific frequency, waveform and peak[147]. Jing *et al*.[136] designed a pulse electromagnetic field generator consists of three identical Helmholtz coils (Figure 8B), it could output different waveforms and parameters.

5.2 The Electric Stimulation Systems

In vivo studies, the modes of electrical stimulations were mainly including invasive, semi-invasive and non-invasive way in bone repair. The invasive way meant of embedding cathode and anode in the injury sites[148,149], and the semi-invasive way meant of embedding the cathode into the injury sites and placing the anode in a cephalad paraspinous locus[150,151]. The non-invasive

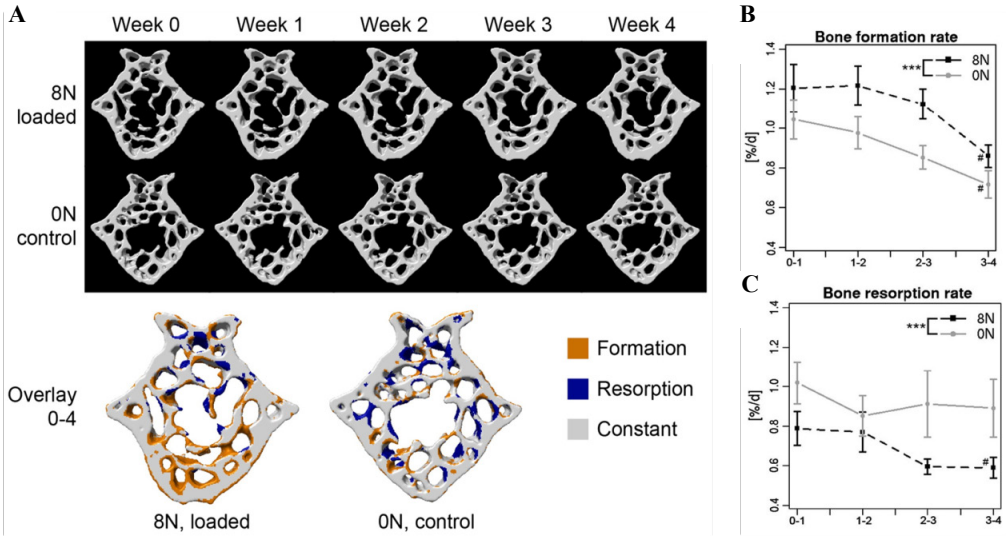

Figure 7. Bone microstructure of mice in the 8 N and 0 N group *in vivo* micro-CT scans (**A**). The trabecular structure of the 8 N group was thickening with increasing stimulation time and had little changes in 0 N group. Curves of dynamic bone formation rate (**B**) and bone resorption rate (**C**) over time. The bone formation rate of 8 N group was obviously higher than 0 N group and the bone resorption rate showed the opposite result.[134]

way referred to place the injury sites in an electric field or place the electrodes on the surface of the treatment sites[152,153]. It had been widely accepted by patients owing to the small trauma. *In vitro* studies, some researchers placed electrodes on top and bottom surfaces of each well and then connected the electric field device to stimulate cells[154]. Many researchers used sinusoidal alternating electric field which consisted of function generator and parallel electrodes to expose bone cells[49,79]. Kim *et al*.[14] designed a biphasic electric current system to stimulate osteoblasts which consisted of Teflon® culture dish, two evaporated Au plates, media and biphasic current stimulator. The anodes and cathodes of each well were connected to form an electrical shunt configuration and all electrodes were connected to the biphasic electric current stimulator chip (Figure 8D). Banks *et al*.[75] customized an ibidi device to create an electric field stimulation system which allowed to simultaneously stimulate six cell migration chambers, and 6 pairs of agar bridges in physiologic buffered saline connected cell migration channels to reservoirs of physiologic buffered saline, in which Ag-AgCl electrodes were immersed (Figure 8E).

5.3 The Mechanical Stimulation Systems

Low intensity pulsed ultrasound treatment had received widespread attention in treatment bone fracture due to the advantages of safety, non-invasive, no infection and less complication[155]. The ultrasonic stimulation used by most researchers was usually generated by the Sonic Accelerated Fracture Healing System, which could produce different frequencies and intensities[81,156,157]. Different devices were used to carry out different mechanical stimulations for bone cells and tissues. The BiopressTM system was used by many researchers to apply compressive loads. It could control load magnitude to compress osteoblasts membrane to investigate the effects of compression stress on osteoblasts [137,158]. Zhong *et al*.[159] designed a cellular cyclic tension and compression apparatus to investigate the biological response of osteoblasts under stretching or compressing. The apparatus could control the carrier rod to precisely shift up and down. Many researchers used a parallel-plate flow chamber to induce fluid flow over the cells to construct fluid shear stress[93,94,160,161]. You *et al*.[162–164] established a flow system which was driven by an electromagnetic actuator.

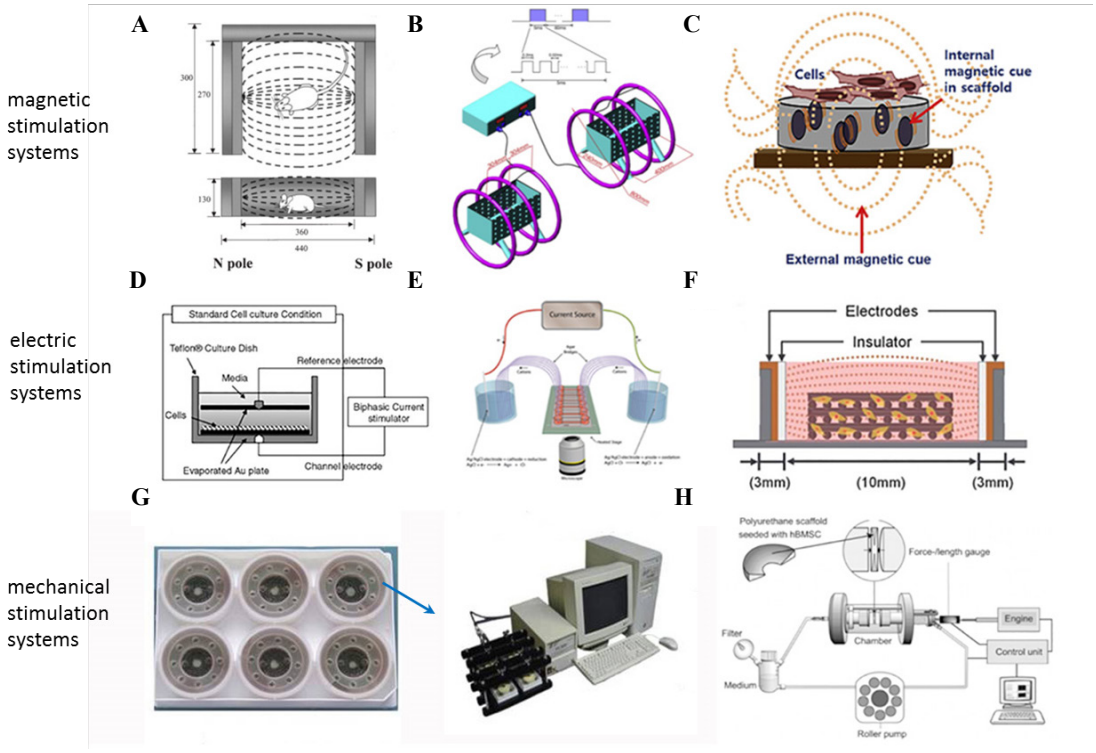

Figure 8. The schematic of the physical stimulation systems. (**A**) static magnetic field exposure system[120]. (**B**) pulse electromagnetic field exposure system[136]. (**C**) Magnetic stimulation on artificial bone[66]. (**D**) Biphasic electric current stimulation system[14]. (**E**) Direct current electric stimulation system[75]. (**F**) Electric stimulation consisted of a parallel electrode used to stimulate cells seeded on scaffold[79]. (**G**) Flexercell compression plus system was used to compress osteoblasts membrane[137]. (**H**) Mechanical stimulation bioreactor system was used to perfuse scaffolds seeded with cells[22].

6. The Opportunities and Challenges of Physical Stimulations

Physical stimulations have been demonstrated to be effective in promoting bone repair. It is urgently require further systematic investigations to find the underlying mechanisms, thereby getting better understanding of the bioeffects and providing adequate theoretical supports for the application in bone repair. Bone is a dynamic tissue composed of several cell types such as osteocytes, osteoblasts, osteoclasts and bone mesenchymal stem cells. The cells play an important role in maintaining normal bone homeostasis. Current researches mainly focus on the bone formation by osteoblasts. Therefore, future researches should comprehensively evaluate the bioeffects of physical stimulations on various cells, and the mutual regulation between cells under physical stimulations should also be considered.

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