stem cell research&therapy

REVIEW

Mechanical control of tissue-engineered bone

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Abstract

Bone is a load-bearing tissue and physical forces play key roles in the development and maintenance of its structure. Mechanical cues can stimulate the expression of an osteogenic phenotype, enhance matrix and mineral deposition, and influence tissue organization to improve the functional outcome of engineered bone grafts. In recent years, a number of studies have investigated the effects of biophysical forces on the bone formation properties of osteoprogenitor cells. The application of physiologically relevant stimuli to tissue-engineered bone may be determined through observation and understanding of forces to which osteoblasts, osteoclasts, and osteocytes are exposed in native bone. Subsequently, these cues may be parameterized and their effects studied in well-defined *in vitro* systems. The osteo-inductive effects of three specific mechanical cues - shear stress, substrate rigidity, and nanotopography - on cells cultured in monolayer or in three-dimensional biomaterial scaffolds *in vitro* are reviewed. Additionally, we address the time-dependent effects of mechanical cues on vascular infiltration and *de novo* bone formation in acellular scaffolds implanted into load-bearing sites *in vivo*. Recent studies employing cutting-edge advances in biomaterial fabrication and bioreactor design have provided key insights into the role of mechanical cues on cellular fate and tissue properties of engineered bone grafts. By providing mechanistic understanding, future studies may go beyond empirical approaches to rational design of engineering systems to control tissue development.

Introduction

Bone tissue engineering (BTE) has the potential to make tremendous clinical impact for the repair and treatment of massive bone loss. While autografts are the current gold standard for treatment, limitations to this approach include tissue availability and donor-site morbidity. Allografts, meanwhile, require the use of immunosuppressive drugs and carry the risk of disease transmission. In contrast, engineered grafts may utilize autologous cell sources with little co-morbidity and can be used to treat critical-sized bone defects. Traditionally, BTE has combined cells with biomaterial scaffolds and osteoinductive biological factors to guide the development of cells into tissue grafts. Initial studies demonstrated cellular expression of bone-specific proteins but the grafts inevitably lacked adequate mechanical properties needed to withstand physiological loads. This shortcoming has been addressed by incorporating biophysical cues into the culture environment. At the most fundamental level, it is critical to understand the mechanism(s) through which cells in native bone are influenced by mechanical cues. Then, guided by the biomimetic principle [1], it may be possible to determine which forces are most effective for developing bone grafts with superior mechanical properties. Even so, knowledge regarding the effect of timing, dose and loading protocols of mechanical stimuli on cells cultured within three-dimensional scaffolds has primarily been determined empirically. Using tissue-culture bioreactors, various biophysical forces have been applied to developing constructs. These forces enhance the expression of an osteogenic phenotype in cells embedded within the scaffold resulting in increased production and organization of the extracellular matrix (ECM) and increased mineral deposition. In this article, we review how our current understanding of the microanatomy of native bone and cellular mechanotransduction has impacted the application of mechanical forces in biomimetic tissue engineering approaches.

Native mechanics of bone

Bone actively and continuously remodels in response to physiological loading. Studies have found that strains experienced by bone tissues due to everyday activity range from 0.1% to 0.35% [2]. Strains above this range (but below the yield point) lead to bone strengthening while sub-physiological strains lead to bone resorption [2-4]. Three major cell types mediate remodeling: osteoblasts (which deposit new bone matrix), osteocytes

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(which are encased in mineral), and osteoclasts (responsible for bone resorption), and it is the coordinated activity of these cells that enable the coupling of bone structure and function. There is evidence that mechanical stimuli influence the proliferation and function of osteoclasts and osteoblasts in a spatiotemporal manner: bone regions experiencing high strains exhibit significant reduction in osteoclast proliferation [5]. Conversely, simulated microgravity conditions have been shown to suppress osteoblast function and numbers [6].

Osteocytes, however, comprise the majority of cells in compact bone, and are the cells primarily responsible for transducing biophysical signals into specific biological responses in bone. The anatomical location of the osteocytes, encased within lacunae, enable them to 'sense' physiological loads. Compressive loading of bone (for example, during walking) results in non-uniform strains macroscopically. The associated volume and pressure differences within the interconnected canalicular network cause interstitial fluid flow, which imparts shear stresses on the order of 1 to 3 Pa to the osteocytes [2,7-9]. This conversion from strain to shear stress amplifies the stimulus received by cells [7] and osteocytes transduce these signals through stretch-activated ion channels [10] and via the primary cilium [11]. As a result, bone cells respond to dynamic stimuli [12,13]; a static load produces an initial pressure gradient, which the resulting fluid flow returns to equilibrium, halting further flow, and abolishing the stimulus. The interconnectivity of osteocytes through canaliculi provides an excellent relay network for transmitting mechanical and biochemical signals to osteoblasts and osteoclasts that reside on the endosteal surface [8]. Exquisite *in vitro* studies, however, have demonstrated that two other physical signals may play a role in directing the activity of osteoprogenitors: the stiffness and topography of the substrate on which they reside. As a result, recent studies regarding the role of mechanics in BTE have focused primarily on the effects of shear stress, substrate rigidity and nanotopography in directing cellular differentiation and enhancing the mechanical competence of engineered bone grafts (Figure 1).

Role of perfusion-mediated shear stress (two- and three-dimensional)

BTE has made extensive use of bone marrow-derived human mesenchymal stem cells (MSCs) and the effects of mechanical cues have primarily been studied using these cells and osteoblastic cell lines. Rigorous studies into the osteogenic effects of shear stress have been performed using monolayer cultures in parallel plate chambers, as these configurations facilitate accurate measurements of actual shear forces experienced by cells. Osteogenic cells exhibit both dose- and time-dependent changes in gene expression in response to shear forces. Comparisons of oscillatory and pulsatile shear profiles in comparison to steady shear stresses revealed that pulsatile shear elicited the greatest osteogenic response [14]. This result is somewhat surprising given that physiological loading and unloading during walking and running result in oscillatory profiles. Other studies [15] have shown that oscillatory shear elicits anti-osteoclastic responses from osteocytes as evidenced by upregulation of cyclooxygenase-2 (COX-2), downregulation of receptor activator of nuclear factor κB ligand (RANKL), and downregulation of osteoprotegerin (OPG). This effect is enhanced at high stress amplitudes (approximately 5 Pa), high frequencies (2 Hz), and long loading durations (4 hours). Interestingly, while each of these parameters alone enhances osteogenesis, it was unclear how they interact. For instance, stress applied at 5 Pa and 0.5 Hz had a greater effect than did stress applied at 5 Pa and 2 Hz. It is clear that further work is required to understand the effect of oscillatory shear parameters on osteogenesis.

Shear stress also impacts cellular behavior in three-dimensional scaffolds. Previous studies have investigated the effects of superficial flow velocities on the osteogenic profile of MSCs grown in porous scaffolds [16-19]. Due to differences in cell types, scaffold types, and bioreactor designs, the results from these various studies cannot be directly compared. It is nevertheless evident that flow velocities can be optimized based on ECM and mineral deposition. An order of magnitude estimation found that shear stress in these systems is likely to be in the mPa range, two to three orders of magnitude lower than reported values for native bone. More rigorous computational fluid dynamics analyses for a similar system reported similar values [20]. Interestingly, subsequent studies also demonstrated correlations between tissue structure and organization within the graft and flow patterns [21].

In spite of these data, it is considerably more challenging to identify the effects of flow-induced shear stress on tissue formation in three-dimensional scaffolds. Firstly, perfusion enhances nutrient and oxygen transport to cells in three-dimensional cultures via convection, making it difficult to decouple the beneficial effects of shear from those of improved mass transport. Additionally, complex flow patterns through the internal scaffold geometry result in complicated shear distribution profiles, making it challenging to correlate specific shear forces with cellular outcomes. This difficulty was partially overcome in three-dimensional systems by varying the medium viscosity while keeping perfusion rates constant. For a given velocity profile, the shear stress is directly proportional to the viscosity of the fluid. Dextran molecules were added to change the viscosity of the flowing fluid while keeping velocity constant, thereby

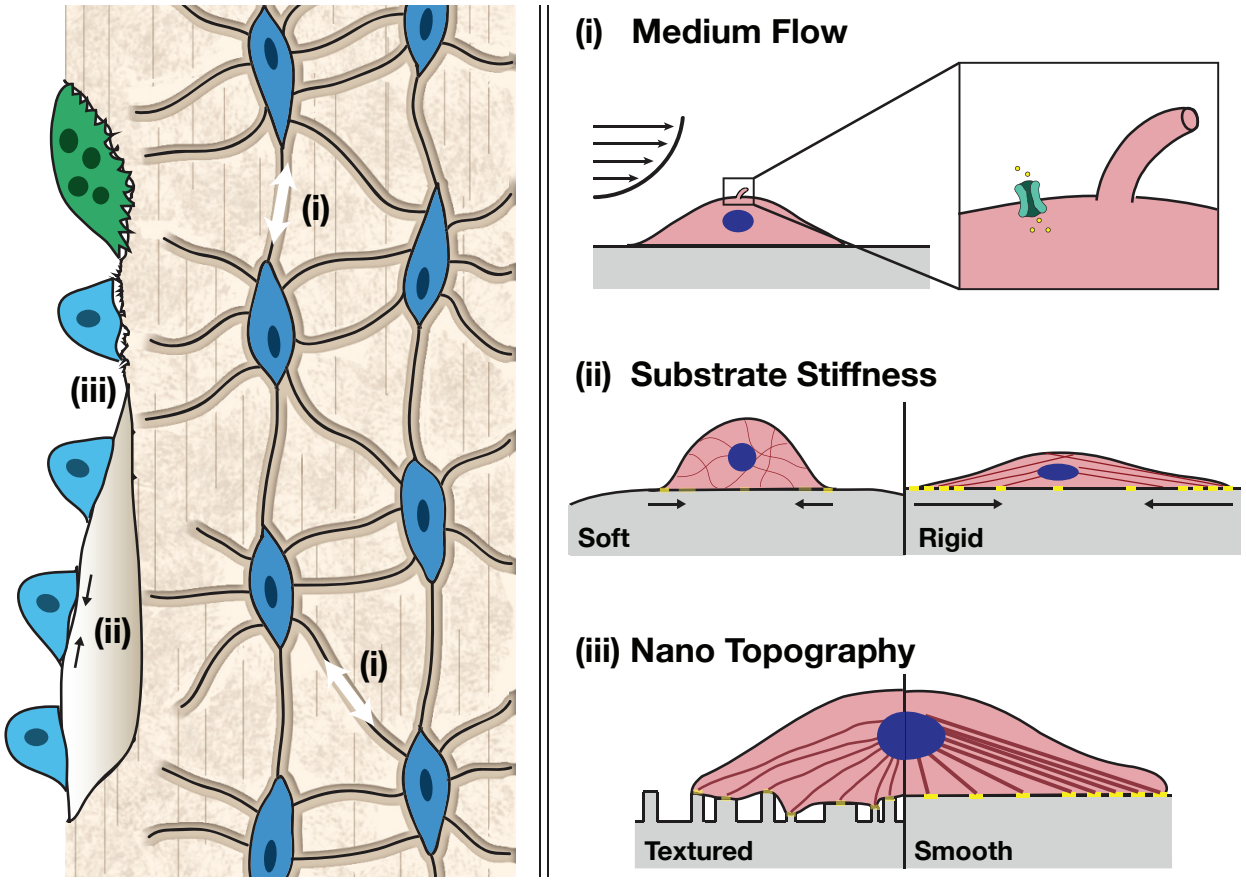

Figure 1. Role of mechanical cues on osteogenic differentiation. Left: native bone. Physiological loading of bone induces fluid flow within the canaliculi, resulting in shear stress on the osteocytes (i), which transmit these signals to osteoclasts and osteoblasts to remodel the bone. Stiffness (ii) and topography (iii) of native bone matrix also impacts new bone deposition by osteoblastic cells. Right: tissue engineers apply mechanical stimuli to enhance the osteogenic response of stem and progenitor cells *in vitro*. **(i)** Perfusion of culture medium over cell monolayers or through three-dimensional constructs imparts shear stress. Shear induces stretching of the cell membrane, allowing an influx of calcium ions through stretch-activated ion channels. Fluid flow also deflects the primary cilia that extend from the surfaces of osteocytes and osteoblasts, altering signal transduction as a result of microtubule tension. **(ii)** Substrate rigidity influences cell adhesion, spreading, and differentiation patterns. Soft surfaces provide low resistance, decreased focal adhesion (yellow) strength and reduced cytoskeletal organization relative to more rigid surfaces. This leads to changes in nuclear shape and gene expression. **(iii)** Topography: surface roughness along with spacing and randomness in nano-scale topographical features influence cell adhesion and the formation of localized stresses along the cell membrane. For example, differences in interfeature z-scale dimensions greater than 50 nm impair local focal adhesion strength. These differences are transmitted to the nucleus via actin filaments and lead to changes in gene expression and cell fate.

increasing shear stress without changing mass transport properties. Grafts exhibited greater matrix and mineral deposition in response to higher viscosity, suggesting that the superior tissue formation characteristics were directly related to increased shear stresses [17]. These three-dimensional studies all employed uniform flow rates for the duration of their culture period. Given the dynamic nature of *in vivo* loads on bone cells, an important area for future studies may be optimizing flow profiles over time (for example, amplitude and frequency of oscillatory and pulsatile patterns) to maximize the formation of new bone.

Scaffold rigidity: role of substrate mechanics

Cells maintain an interactive, bi-directional signaling relationship with their immediate microenvironment, modifying and organizing the ECM while also directly responding to a plethora of cues provided by the ECM. The biochemical and physical features of the ECM, including the composition and concentration of adhesive ligands, topography, and rigidity impact cellular physiology and influence cell shape, motility, polarization, and cytoskeletal alignment through the formation of focal adhesion complexes. Upon binding the ECM, cells exert contractile forces. The resistance to these forces provides

information to the cells regarding the compliance of the underlying substrate. Pioneering studies have demonstrated that the rigidity of polyacrylamide gel substrates critically influences the differentiation of MSCs [22]. In particular, MSCs cultured on substrates with elastic moduli mimicking those of brain, muscle, or non-mineralized bone tissues responded by adopting the phenotypic characteristics of neuronal, myogenic, or osteogenic lineages, respectively. The expression of bone markers was highest when MSCs were cultured on the stiffest gels having elastic moduli of approximately 100 kPa, which is similar to that measured for non-mineralized bone. It should be noted, however, that while induction media containing traditional biochemical factors directed cells towards a specific lineage only when cultured on substrates within the optimal ranges of stiffness for that tissue, it appeared that substrate stiffness was not itself a sufficiently potent cue to guide undifferentiated stem cells down a given lineage.

Similar results have been reported regarding the osteogenic and adipogenic potential of MSCs: using a system of micrometer-scaled pillars, it was possible to independently regulate cell adhesion (focal adhesion density) and substrate stiffness by controlling the spacing and height, respectively, of the pillars [23]. MSCs cultured in this system were exposed to cocktails of adipogenic and osteogenic factors. It was found that softer surfaces induced a greater adipogenic response, while stiffer surfaces stimulated osteogenic differentiation of cells. The results of both studies conclusively demonstrate that physical characteristics of the matrix environment are critical for the adoption and maintenance of cellular phenotype.

The results from these monolayer studies indicate that it is important to consider the mechanical properties of biomaterial scaffolds used for stem cell-based BTE. Recent studies with silk scaffolds by independent groups have demonstrated that scaffold stiffness influences the composition and mechanics of the resulting tissue grafts. Silk fibroin has been extensively used for tissue engineering applications [24]. It is a naturally derived, biodegradable material and has tunable mechanical properties [25]. In a study using adipose-derived stem cells seeded into scaffolds of different stiffnesses, mechanical tests after 7 weeks of osteogenic culture indicated that the scaffolds with the highest initial stiffness also induced the highest increase in mechanical properties. Similar results were reported in a study using silk scaffolds reinforced with silk microparticles [26]. In this case, MSCs seeded into the stiffest scaffolds exhibited the highest calcium content and expression of bone-specific proteins, but not the greatest increase in bone volume fraction as determined using micro-computed tomography.

While these results demonstrate the impact of scaffold mechanics on the cellular responses, the ultimate mechanical properties of the resulting tissue grafts remain suboptimal for bone applications. For example, the maximum modulus achieved by silk scaffolds after *in vitro* culture was around 150 kPa while the modulus of native bone may be several orders of magnitude higher. Hence, *in vivo* applications typically use stiffer scaffolds made of β-tricalcium phosphate (β-TCP) [27,28], hydroxyapatite (HA) [29,30], or even combinations of the two [31]. Incorporating HA into the wall structure of silk scaffolds significantly enhanced the bone tissue formation properties of MSCs cultured *in vitro* [32]. In this case, HA impacted both the stiffness and biochemical composition of the scaffold. The resulting mineralization structure, however, strongly suggested that the increased wall roughness played an instrumental role in guiding mineral deposition with the HA 'nodes' on the surfaces effectively acting as 'nucleation sites'. Additional studies have demonstrated that topography may also provide mechanical signals that can be transduced directly by cells and influence a number of key cellular processes, including adhesion, contact guidance, cytoskeletal assembly, and gene expression [33].

Mechanical effects of surface topography

Recent advances in fabrication techniques enable the formation of nano- and micro-scale structural components to study their effects on cellular outcomes. Nanotopographic cues such as pores, ridges, pits, islands, grooves, fibers, and nodes can elicit cell type-dependent behaviors with features as small as 10 nm. Using colloidal lithography to control the application of cylindrical features (100 nm diameter, 160 nm height, and spaced 230 nm apart [34]), it was demonstrated that nanotextured substrates limit cell spreading and cytoskeletal organization by inhibiting the formation of robust and dense focal adhesions, resulting in decreased tension on the cytoskeleton. Forces transmitted to the nucleus via the cytoskeleton induce changes in nuclear deformation leading to altered gene expression [35,36].

Consequently, nanomaterials have exhibited considerable ability to regulate cell differentiation and tissue formation characteristics [37]. One landmark study reported that simply by providing disorder to the nano-scaled pillars, it was possible to enhance the expression of osteopontin and osteocalcin in MSCs even in the absence of osteogenic supplements in the culture medium. Cells were cultured on square, hexagonal, disordered (pillars displaced from their position in a square), and random patterned surfaces. Intriguingly, it was found that highly ordered patterns were inhibitory to osteogenesis while displacing the pillars approximately 50 nm from their ordered geometry enabled statistically significant increases

in the expression of osteo-specific genes [34]. Other studies have also investigated the effect of cell shape on MSC osteogenic capabilities. A recent study utilized micro-patterned substrates to regulate MSC adhesion and spreading [38]. As a result, BMP-induced osteogenesis was inhibited. This suggests that cellular responses to nanotography might either be directly due to mechanotransduced signals or may be indirectly related to alterations in biological responses due to changes in cell shape.

These reports indicate an additional mechanism for controlling stem cell differentiation and tissue formation properties. They can provide alternatives to invasive inhibition studies to investigate fundamental biological questions. The knowledge gleaned from these studies may then be applied to enhance biomaterials used for regeneration. For example, fibrous capsules often surround bone prostheses and prevent their direct integration with bone tissues. High throughput assays may enable deeper understanding of cell-material interactions and provide insight into how materials might be altered to optimize integration with the host tissues [39].

Mechanical regulation of bone growth *in vivo*

Upon transplantation into a host, a milieu of cellular and biochemical factors impact the viability of engineered bone grafts. This complex microenvironment, which includes inflammatory and neo-vascularization responses, significantly affects stem cell differentiation and shapes tissue formation patterns. Additionally, bone grafts implanted into load-bearing sites are subjected to physiological loading. Regulating the temporal (immediate versus delayed) application of these loads affects graft-host integration and impacts tissue formation profiles. A widely studied model of mechanics in bone graft regeneration is the femoral defect in rats. In a study investigating the treatment of 8 mm defects in rat femurs, a modified alginate scaffold was implanted and engineered to provide controlled release of bone morphogenetic protein 2 (BMP-2). The approach relied on recruitment of the host's osteoprogenitor cells in response to the released growth factor. Internal fixation plates were used to maintain the alignment of the femur. Based on their design and compliance, the plates (i) shielded the grafts from mechanical loads for the entire 12-week implant period, (ii) transferred load to the graft immediately after implantation, or (iii) only after the first 4 weeks of implantation. It was shown that immediately exposing the grafts to sustained physiological loads resulted in scaffold failure by 12 weeks post-implantation. Alternatively, shielding the graft for 4 weeks before exposing them to physiological loads for the subsequent 8 weeks improved bone volume and integration with host tissue relative to the control group (shielded for the duration of the study). These results show the complexity of tissue outcomes in response to temporal mechanical control [40]. In a subsequent study, the identical defect model was used to demonstrate the effect of mechanics on the interplay between bone formation and angiogenesis [41] into the scaffold and provide mechanistic insight into earlier results. Allowing the scaffold to withstand physiological loading immediately upon implantation inhibited vascular ingrowth and subsequent osteogenesis. Alternatively, shielding the scaffolds from loading for the first 4 weeks post-implantation allowed the infiltration of neo-vasculature. Increasing the compliance of the plate at this time allowed invading osteoprogenitor cells to respond to mechanical stresses, leading to an overall enhanced endochondral ossification response compared to control groups [42]. This result corroborates earlier studies where structures resembling secondary ossification centers appeared in the explanted femoral condyles of 5-day old rabbits after exposure to cyclic mechanical loading at 1 Hz for 12 hours [43]. The potential for using mechanical cues to inhibit bone formation has also been studied using a 1.5 mm transverse defect in the rat femur [44]. Application of cyclic bending beginning at 10 days post-operation resulted in slowed bone healing and increased cartilage volume, evidenced by histological staining for Safranin O and gene expression data for cartilage markers collagen II and collagen X. This is consistent with studies demonstrating that the increased cartilage production is actually a prolonged cartilage phase in an endochondral ossification process [45,46].

Conclusion

Bone tissue engineering makes considerable use of insights from mechanobiology studies and many advances have been made in utilizing mechanics to improve the functionality of bone grafts. Understanding the anatomical structure of native bone and how forces are transmitted to cells has revealed the need to implement fluid-induced shear stress, substrate compliance and topography as biophysical stimuli integral to bone tissue engineering. In three-dimensional *in vitro* systems, scaffolds typically shield cells from the direct effects of compressive forces, so compression is rarely used to enhance osteogenic outcomes *in vitro*. However, during fracture healing *in vivo*, compression may work synergistically (and in a time-dependent manner) with other microenvironmental stimuli, to enhance bone formation via an endochondral ossification pathway.

Scaffold stiffness has profound effects on the osteogenic differentiation of MSCs and *in vitro* studies have revealed that cells respond to more rigid scaffolds by increasing mineral deposition. Continuing, conventional wisdom suggests that it is desirable to replace 'like with like';

hence, bone grafts should have mechanical properties approximating that of native bone to provide immediate functionality upon implantation. The validity of this assumption, however, remains debated as, reportedly, rigid scaffolds do not integrate as readily with host tissues as softer grafts. In examining the role of mechanics on *in vivo* bone repair, it is not universally accepted that exogenous cells are required and the roles of these 'endogenous' approaches to bone repair have received increasing attention [47].

Future BTE studies will continue to incorporate mechanical considerations to enhance osteogenic differentiation and mineral deposition within grafts. More fundamental understanding of mechanotransduction is nevertheless required to overcome empirical approaches. Non-invasive image-based modalities used to study *in vivo* bone formation processes in response to specific mechanical stimuli [48] would help to integrate mechanics with other important parameters capable of influencing bone development.

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Abbreviations

BMP-2 bone morphogenetic protein 2; B-TCP-B-tricalcium phosphate; BTE, bone tissue engineering; COX-2, cyclooxygenase-2; ECM, extracellular matrix; HA, hydroxyapatite; OPG, osteoprogerin; RANKL, receptor activator of nuclear factor kB ligand.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

BPH, DLH and WLG wrote the paper, conceptualized and drew the schematic. All authors read and approved the final manuscript.

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