Mechanobiology of TGFβ Signaling in the Skeleton

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Abstract

Physical and biochemical cues play fundamental roles in the skeleton at both the tissue and cellular levels. The precise coordination of these cues is essential for skeletal development and homeostasis, and disruption of this coordination can drive disease progression. The growth factor TGFβ is involved in both the regulation of and cellular response to the physical microenvironment. It is essential to summarize the current findings regarding the mechanisms by which skeletal cells integrate physical and biochemical cues so that we can identify and address remaining gaps that could ultimately improve skeletal health. In this review, we describe the role of TGFβ in mechanobiological signaling in bone and cartilage at the tissue and cellular levels. We provide detail on how static and dynamic physical cues at the macro-level are transmitted to the micro-level, ultimately leading to regulation at each level of the TGFβ pathway and to cell differentiation. The continued integration of engineering and biological approaches is needed to answer many remaining questions, such as the mechanisms by which cells generate a coordinated response to physical and biochemical cues. We propose one such mechanism, through which the combination of TGFβ and an optimal physical microenvironment leads to synergistic induction of downstream TGFβ signaling.

Overview

Skeletal extracellular matrix

The unique mechanical behavior of each skeletal tissue is regulated in part by its unique extracellular matrix (ECM). Among their many essential roles, tissues such as bone, cartilage, skeletal muscle, ligament, and tendon routinely encounter mechanical forces as part of their structural, locomotor, and protective functions. Given this diversity, this review focuses on bone and cartilage. Insights into the mechanobiology of bone and cartilage inform our understanding of less-extensively studied skeletal tissues. Lest we view the skeleton as the static infrastructure of the body, this ECM is dynamic and biologically regulated. For example, changes in either metabolism or mechanics profoundly impact bone mass and quality [1,2]. Likewise, biological and physical cues are able to direct the composition and organization of the ECM of bone, cartilage, tendon, and other musculoskeletal tissues.

Recent advances in cellular mechanobiology highlight the role of transforming growth factor-beta (TGFβ) in mediating a cellular response to physical cues via a feedback loop (Figure 1). On one side, TGFβ regulates ECM synthesis and remodeling that can specify the material quality of the ECM and help coordinate cytoskeletal tension [3,4]. Cytoskeletal tension, in turn, regulates the TGFβ pathway at several hierarchical levels, including transcription, translation, ligand activation, receptor multimerization, effector selection, and expression of downstream lineage-specific transcription factors. These transcription factors bind to promoters of TGFβ-regulated lineage-specific ECM proteins. Through these mechanisms, TGFβ signaling continuously balances cellular mechanical integrity with ever-changing physical demands [5].

This review focuses on TGFβ in the mechanobiological mechanisms by which skeletal cells and their ECM integrate physical and biochemical cues to support bone and cartilage function. These mechanisms are essential for skeletal homeostasis and their deregulation contributes to diseases ranging from post-traumatic osteoarthritis to bone fragility, both of which have been integrally linked to defects in TGFβ signaling [6-8]. This mechanistic understanding has the potential to reveal novel molecules and pathways that can be targeted therapeutically to improve skeletal health.

TGFβ signaling in the skeleton

TGFβ is the prototype of a large family of growth factors that also includes bone morphogenetic proteins (BMPs), activins, and growth differentiation factors (GDFs). In this review, "TGFβ" is used generically to refer to any of the 3 TGFβ ligands or the TGFβ pathway except when the use of TGFβ1, 2, or 3 in a specific study is noted. Like other family members, TGFβ itself regulates diverse cellular behaviors ranging from fate specification, lineage selection, and differentiation, to epithelial-mesenchymal transition, migration, proliferation, and apoptosis [9]. At a high level, TGFβ signals through a complex of heterotetrameric transmembrane receptor serine/threonine kinases. Once the TGFβ ligand is activated from its latent form – for example via integrin-mediated activation, cytoskeletal tension, or acid- or protease-mediated cleavage – it binds directly to a pair of type II receptors (TβRII) [10-14]. The ligand-bound TβRII complex recruits and phosphorylates two type I receptors (TβRI) – either Alk5 or Alk1 [15,16]. TβRI, in turn, phosphorylates and activates Smad2/3 proteins and multiple non-canonical effectors, such as Smad1/5/8, RhoA, TAK1, and Akt [17,18]. In complex with Smad4, phosphorylated Smads translocate to the nucleus where they interact with sequence-specific transcription factors, coactivators, and corepressors to modulate gene expression.

TGFβ activity is dependent both on cell-intrinsic factors, such as the composition of cell surface receptor complexes or the availability of specific transcription factors, and cell-extrinsic factors, such as the activity of other signaling pathways or the physical features of the ECM [19]. The effect of these cell-intrinsic and cell-extrinsic factors on TGFβ signaling underlies the exquisitely context-dependent behavior of this growth factor. However, this complexity can be difficult to unravel experimentally and must be considered carefully, especially when comparing *in vivo* and *in vitro* findings. One example of this complexity is the TGFβ-mediated control of mesenchymal differentiation, which depends in part on TGFβ/Smad3 action on various lineage-specific transcription factors. TGFβ-activated Smad3 promotes chondrogenic differentiation by facilitating recruitment of the coactivator CREB-binding protein (CBP) to transcriptional complexes containing the chondrogenic transcription factor Sox9 [20,21]. On the other hand, TGFβ inhibits osteogenic differentiation through Smad3 recruitment of the corepressor histone deacetylase 4 to repress Runx2-inducible osteogenic gene expression [22,23]. TGFβ also promotes differentiation of mesenchymal progenitors into the tenogenic lineage, again by

targeting lineage specific transcriptional regulators such as Scleraxis and Mohawk [24]. As will be discussed later, cell-extrinsic differences in ECM stiffness or topography further influence the ability of TGFβ to promote chondroinduction of mesenchymal progenitors (MSCs) [25]. Thus, the combination of cell-intrinsic factors and cues presented in the cellular microenvironment dramatically alter the activity of the TGFβ pathway in skeletal cell differentiation.

Another key function of TGFβ is its ability to control ECM synthesis and remodeling. TGFβ regulates the expression of various ECM proteins, such as fibronectin, collagens, and other matrix glycoproteins [9]. Depending on the cell type and context, TGFβ also controls the expression of proteases such as matrix metalloproteases (MMPs) and their inhibitors (TIMPs) [26,27]. In this way, TGFβ can stimulate or limit ECM remodeling; however, the ECM, in turn, also regulates TGFβ signaling. The heparan sulfate domains of many ECM proteins such as fibronectin bind and sequester TGFβ in the ECM [28]. In addition, TGFβ sequestration via the small leucine-rich proteoglycans biglycan and decorin helps coordinate bone marrow stromal cell fate [29]. Not only do these protein/protein interactions provide spatial control of ligand availability, but they also regulate the activation of latent TGFβ. The ECM of bone, in particular, has high local concentrations of TGFβ [30].

In part because of its role in regulating skeletal cell differentiation and ECM synthesis, TGFβ plays a vital role in the development and homeostasis of many skeletal tissues. TGFβ, let alone the other TGFβ family members, has been implicated in over one dozen human skeletal diseases, most recently in the bone fragility associated with osteogenesis imperfecta [6,31]. Many *in vitro* and *in vivo* studies have elucidated cellular and molecular mechanisms that underlie these actions. This insight has motivated the development of pharmacologic agents to manipulate TGFβ signaling therapeutically. Several clinical trials are currently exploring the utility of these agents for a variety of conditions, including those in the skeleton [32]. Because of the scope of this topic, we refer readers to other articles that review the important role of TGFβ signaling in the skeleton and in skeletal disease [3,31,33,34]. Here we focus on the role of TGFβ in the regulation of skeletal ECM and in the response of skeletal cells to physical cues.

Multi-scale mechanobiology of the skeleton

Distinct features of the ECM, comprised of fibers and ground substance, support the mechanical function of each skeletal tissue. Almost all skeletal tissues utilize collagen fibers to provide toughness and resistance to tension, most notably the tendons and ligaments. The ground substance of skeletal extracellular matrices is more variable. Bone, dentin, and enamel rely on mineral to resist plastic deformation, whereas cartilage and intervertebral disc employ proteoglycans for this function. Progress in understanding the mechanobiology of bone and cartilage can serve as a foundation for more detailed analyses of tendon, dentin, intervertebral disc, and other less well-studied skeletal tissues.

Each skeletal tissue senses and responds to physical cues at multiple hierarchical scales. At a basic level, locomotion produces macromechanical forces that bone experiences as compressive and tensile strains, depending on the specific local geometries of each bone [35]. The same motion produces compression and tension in cartilage. The fibers and ground substance of the skeletal ECM and skeletal cells respond to these forces in a variety of ways. For example, skeletal loading forces fluid through canalicular networks in bone that osteocytes sense as shear flow [36]. Upon compression of cartilage, water is depleted from the proteoglycan-rich ECM, resulting in osmotic pressure changes in chondrocytes [37-39]. Tenocytes experience stretch-induced changes in cytoskeletal tension [40].

Even at rest, changes in the material properties and organization of the ECM alter cytoskeletal tension. Through actomyosin contractility, cells generate cytoskeletal tension by pulling on the ECM at integrin-rich focal adhesions. This process initiates a host of molecular responses which reveal the effect of physical cues at the molecular scale, for example by stretching proteins to expose hidden domains that alter binding or enzymatic activity [41,42]. These changes impact cellular behaviors from migration to differentiation [43]. Critical studies examining skeletal differentiation of MSCs revealed the profound effect of ECM stiffness and shape on lineage selection [44,45]. For example, McBeath et al. showed that substrate shape and cell spreading directs lineage selection between osteoblast or adipocyte fates by modulating Rho activity [46]. Several additional studies have since drawn related conclusions.

Micrometer-sized topographical features, such as the roughness of a titanium surface, influence cell behaviors including osteoblast attachment and the expression of osteoinductive transcription factors [47]. Changes in cytoskeletal tension resulting from topographic features can elicit responses similar to those induced by changes in the stiffness of the cell substrate. Dalby et al. further demonstrated that osteoprogenitors on disordered nanoscale features preferentially expressed bone-specific ECM proteins, osteopontin and osteocalcin, and formed bone nodule-like structures [48]. Such microstructures also regulate chondrocyte proliferation [49]. Furthermore, these physical cues alter the cellular response to growth factor signaling, for example by enhancing the chondroinductive effects of TGFβ [25,50]. Thus, physical cues intersect with biological systems at each of these length scales.

Additional studies that span these length scales are needed to help answer fundamental questions. Among them are "What are the mechanisms by which cells discriminate among the many types of macromechanical cues present in the skeleton?" and "How do cells integrate signaling by physical and biochemical cues?". Disciplinary gaps present challenges to finding these answers, in part because our insight into the macro-level derives more heavily from engineering and materials science, whereas cellular biology and biophysics inform our understanding of molecular scale mechanobiology. Therefore, this review seeks to provide macromechanical context for mechanobiological observations in skeletal biology.

Tissue-level roles of TGFβ in skeletal mechanobiology

Like TGFβ, physical cues influence skeletal tissue development, homeostasis, and disease. As detailed below, some important studies have elucidated mechanosensitive, TGFβ-dependent mechanisms that are involved in these processes. Many others are consistent with the idea that the mechanoregulation of TGFβ is involved in bone or cartilage homeostasis or skeletal disease. However, for the most part, these causal relationships remain to be established. Therefore, an improved macroscale understanding of the coupling of physical cues and TGFβ in skeletal health or disease has important therapeutic implications.

Bone

Bone exists in a state of dynamic equilibrium, undergoing competing processes of formation and resorption. Crosstalk among osteoblasts, osteoclasts, and osteocytes maintains bone mass even in the face of changing mechanical or metabolic demands [1,2]. The most well-defined mechanism by which mechanical loads stimulate an increase in bone mass reveals the critical role of the Wnt pathway [51,52]. Mechanical load represses osteocyte expression of sclerostin, a secreted antagonist of the osteoinductive Wnt signaling [53]. Although much more is known about the mechanosensitivity of Wnt signaling in bone, TGFβ also plays a critical role in the anabolic response of bone to mechanical load. For example, ablation of TGFβ receptors prevents load-induced bone formation and repression of sclerostin expression [54]. This occurs in part through mechanosensitive regulation of Smad3 phosphorylation. Interactions between the TGFβ and Wnt signaling pathways are known to occur at multiple hierarchical levels, including ligand production, effector crosstalk, and regulation of shared target genes [55]. Thus, mechanical load regulates the activity of the two key pathways that regulate bone homeostasis - TGFβ and Wnt - through mechanisms that are coupled but remain to be fully elucidated.

Many other factors in addition to bone mass influence the ability of bone to resist fracture. These factors, collectively considered 'bone quality', include bone geometry, trabecular microarchitecture, and ECM material properties, among others [56]. The material properties of bone ECM are site-specific, biologically regulated, and functionally essential, and are controlled by TGFβ signaling through a TGFβ-, TβRI/TβRII-, Smad3-, and Runx2-dependent mechanism [37]. They can be regulated postnatally by pharmacologic antagonists of TGFβ signaling [38,39]. This TGFβ-dependent control of bone quality may contribute to the fragility in patients with osteogenesis imperfecta, in which collagen mutations deregulate the activity of the TGFβ pathway [6]. Though TGFβ is clearly mechanosensitive in bone and in other tissues, the extent to which bone quality is mechanoregulated through a TGFβ-dependent pathway remains to be determined.

Articular Cartilage

Articular cartilage serves as a viscoelastic, lubricated cushion allowing for joint articulation with minimal wear. Though 80% water, the cartilage ECM is mainly comprised of proteoglycans such

as aggrecan, hyaluronic acid, and both fibrillar and non-fibrillar collagens. The integrity of articular cartilage ECM is regulated in part by mechanical loading. While healthy loads promote cartilage homeostasis, excessive loads can also be harmful [57]. During loading, water is forced out of the ECM, imparting direct strain to chondrocytes in addition to generating secondary physical cues such as fluid shear stress and hydrostatic and osmotic pressures [58-60].

Chondrocytes respond to these physical cues through multiple mechanisms including ion channels, focal adhesions, and primary cilia [61-63]. For example, O'Conor et al. identified TRPV4 as an osmotically sensitive transducer of mechanical loading that induces TGFβ3 gene expression and cartilage ECM synthesis [64]. Other known mechanotransduction participants, integrins and primary cilia, are important regulators of growth factors signaling in cartilage. Integrins modulate mechanosensitive chondroinduction by TGFβ [25,65], and primary cilia support the chondrocyte response to hedgehog signaling [66]. TGFβ receptors also localize to primary cilia, but the role of ciliary TGFβ signaling in mechanotransduction remains to be determined. Deregulation of either TGFβ or hedgehog signaling has been implicated in osteoarthritic degeneration of articular cartilage [7,67].

Osteoarthritis

Disruption of TGFβ signaling through Smad3 has been causally implicated in human osteoarthritis [68]. *In vivo* and *in vitro* studies reveal that a chondrocyte-specific reduction of Smad3 drives articular cartilage degeneration due to an imbalance between cartilage matrix synthesis and degradation [69]. This protective role for TGFβ in articular cartilage is also apparent in studies inhibiting endogenous TGFβ signaling via injection of soluble TβRII ectodomain or by overexpression of TGFβ antagonists such as LAP or Smad7. Inhibition of TGFβ interfered with cartilage repair, but it also prevented osteophyte formation [70,71]. Interestingly, TGFβ1 concentrations are elevated in osteoarthritic subchondral bone, and inhibition of TGFβ signaling in subchondral bone MSCs actually attenuates osteoarthritis [72]. This suggests that the dual role of TGFβ in the progression of osteoarthritis is, in part, related to its tissue-specific effects on bone and cartilage of the joint. Another factor is the balance of canonical and non-canonical effectors activated by TGFβ. While TGFβ signaling through Alk5 activates Smad2/3 to promote chondrocyte homeostasis, signaling through Alk1/Smad1/5/8 or

p38/MAPK drives an arthritic phenotype. With age, chondrocytes express higher levels of Alk1 [73]. Furthermore, Smad2/3 phosphorylation is induced by mechanical loading in young cartilage, but this mechanosensitive activation of Smad2/3 is impaired in aged cartilage [74]. As cartilage degrades and the biochemical composition and structure of the ECM are altered, the material properties of cartilage, including its elastic modulus, also change [75]. The extent to which these physical changes in the cellular microenvironment alter the activation of canonical and non-canonical TGFβ effectors or exacerbate the loss of chondrocyte homeostasis in arthritis remains to be determined. Overall, however, it is clear that the mechanoregulation of TGFβ signaling plays a critical role in cartilage homeostasis and the deterioration of cartilage with age and osteoarthritis. Additional research into these mechanobiologic mechanisms is needed to improve our understanding of and ability to prevent or treat this widespread debilitating disease. Already this insight has been applied to advance the use of stem cells for cartilage tissue regeneration, as discussed briefly below.

Skeletal development and regeneration

Physical cues are essential for the development and maintenance of skeletal tissues. Embryonic muscle contractions or maternal movements modulate skeletal development to define bone size and shape and joint cavitation [76]. Mechanical forces from embryonic muscles promote the developmental integration of muscle, tendon, and bone at tendon insertions [76-78]. Postnatal mechanical forces also participate in defining bone shape, as exemplified by the increasing angle of the femoral head relative to the diaphysis in humans throughout early childhood [79]. Insight derived from the study of physical cues in skeletal development and homeostasis has been applied practically to promote the directed differentiation of progenitor cell populations for skeletal tissue regeneration [80]. A common approach has been to compare chondroinduction of stem cells in response to inductive physical and biochemical cues, both alone and in combination. This combination of cues often has a synergistic effect on chondrocyte differentiation and articular cartilage ECM synthesis. For example, treatment of bovine cartilage explants with hydrostatic pressure and TGFβ increased Young's modulus and collagen content over levels resulting from application of hydrostatic pressure or treatment with TGFβ alone [81]. While it is clear that physical cues can prime cells for a more robust response to growth factor

stimulation, the mechanisms by which cells integrate these diverse stimuli remain to be determined.

Cellular and molecular roles of TGFβ in skeletal mechanobiology

The effect of static and dynamic physical cues on TGFβ signaling has been examined in multiple tissues, including in the skeleton. Collectively, these studies reveal the mechanosensitive regulation of the TGFβ pathway in the skeleton from ligands and receptors to transcription factors in the nucleus (Table 1). These mechanisms have proven relevant for diverse cell types in and out of the musculoskeletal system and represent molecular solutions to the fundamental cellular challenge of integrating diverse biochemical and physical cues to generate a coordinated cellular response.

TGFβ mRNA and protein expression

The expression and activity of the TGFβ ligands are sensitive to a wide variety of physical stimuli. It has long been established that physical cues regulate TGFβ1 expression at the mRNA and protein levels in both cartilage and bone. In 1994, Raab-Cullen et al. found that the application of mechanical load to tibial periosteal bone rapidly induces TGFβ mRNA levels [82]. Sakai et al. demonstrated that physiological levels of fluid shear stress increase TGFβ1 protein expression in osteoblast-like Saos-2 cells [83]. Because osteocytes sense shear stress following macromechanical load, the effect of shear stress on TGFβ expression helps to couple physical and biological signals with the control of bone remodeling. In cartilage, cyclic compression on hMSCs in scaffolds stimulates mRNA and protein expression of TGFβ1 and TGFβ3, both of which can promote chondrogenesis [84]. Static physical cues such as ECM stiffness, shape, or topography also regulate TGFβ1 ligand expression. On an inductive substrate stiffness that promotes chondrogenic gene expression, TGFβ1 mRNA expression is also induced. Inhibition of this autocrine TGFβ1 significantly blunts the chondroinductive effects of substrate stiffness [25]. TGFβ1 expression is also sensitive to topography cues in bone implants, such that production is higher on rough surfaces than on smooth surfaces [85]. Therefore, regulation of TGFβ ligand expression is a key mechanism by which cells respond to physical cues.

Activation of latent TGFβ ligand

Not only is the TGFβ ligand regulated at the transcriptional level, but it is also undergoes post-translational control. TGFβ is synthesized with large prodomains, necessary for folding and dimerization [86]. Before secretion, this larger proprotein undergoes cleavage to yield the TGFβ ligand and a latency associated peptide (LAP). TGFβ and LAP interact non-covalently to yield the small latent complex (SLC) [87]. Secretion of the SLC occurs after this complex has itself been bound by latent TGFβ-binding protein (LTBP), which inactivates the ligand and enables it to remain sequestered by the ECM. This large latent complex (LLC) remains inactive in the ECM until a physical or biochemical cue enables the release of TGFβ. This manner of storing latent TGFβ essentially provides cells with a reservoir of TGFβ in the ECM [88]. While these mechanisms are most well-defined for TGFβ ligands, the activity of other TGFβ family members is also regulated through post-translational mechanisms, resulting in their ECM sequestration. As one example, new genetic data has implicated a role for fibrillin 2 in the regulation of latent BMP [89].

The mechanisms that activate latent TGFβ are cell type- and context-dependent. For example, in bone, the osteoclast-derived acidic microenvironment that facilitates bone matrix resorption disrupts the interaction between LAP and TGFβ to activate TGFβ [14]. Tissue-specific expression of thrombospondins, which also induce TGFβ activation, provides local control of TGFβ latency and activation [90]. The TGFβ prodomains contain an RGD motif that is recognized and bound by integrin αv [86]. Integrin-mediated activation of TGFβ can occur through both protease-dependent and protease-independent mechanisms [87]. Critical studies in myofibroblasts revealed that in protease-independent TGFβ activation, cell-generated traction forces are transmitted through integrins bound to latent TGFβ1 sequestered within a rigid ECM [13,91]. These forces induce a conformational change that releases TGFβ from LAP, allowing it to bind to TGFβ receptors [13].

The extent to which tension-dependent activation of latent TGFβ operates in other skeletal cell types remains to be documented. Nonetheless, important findings in cartilage and tendon suggest the role of similar mechanisms. Albro et al. demonstrated that mechanical shearing of synovial fluid activates a substantial amount of latent TGFβ, which then remains stable in synovial fluid

[92]. Maeda et al. found that tendon transection *in vivo* increases activated TGFβ levels and posit that this sudden interruption of tensile loading might destabilize ECM structure, releasing active TGFβ [93]. This mechanism would complement the increases in TGFβ expression or secretion in response to stretch or fluid flow in tenocytes [93,94]. In bone, deregulation of latent-TGFβ activation results in Camurati-Engelmann disease [95-98]. Therefore, it will be especially important to determine the extent to which these mechanosensitive mechanisms of latent TGFβ activation occur in bone at the tissue or cellular level.

TGFβ signaling at the receptor level

For TGFβ and for other signaling pathways, the regulation of receptor clustering, multimerization, and internalization affects ligand binding and effector recruitment as well as downstream signal intensity and duration [99-102]. For example, the internalization and endocytosis pathways of TGFβ receptors influence receptor function and activity [100]. Receptor internalization through clathrin-coated pits promotes downstream TGFβ activity, as the Smad anchor for receptor activation (SARA) is enriched. In contrast, internalization through lipid raft-caveolar pathways leads to receptor degradation by interactions with Smad7-Smurf2 [103]. TGFβ receptors have also been shown to interact with integrins, a key component of the mechanotransduction pathway, such that an active integrin β1 subunit is required for collagen-induced Smad activation [104]. Nonetheless, a direct link between physical cues and TGFβ receptor organization and activity has only recently been established.

We recently demonstrated for the first time that cytoskeletal tension regulates the spatial organization and multimerization of TGFβ receptors at sites of cellular focal adhesions in ATDC5 chondroprogenitor cells [65]. Specifically, focal adhesions discretely organize TGFβ receptors such that TβRI is included within adhesions and TβRII is excluded from these sites. Disruption of cytoskeletal tension through the use of chemical inhibitors or culturing cells on compliant substrates releases this highly-structured organization and drives TβRI/TβRII heteromerization, leading to an increase in downstream effector Smad3 phosphorylation. Visualization using TIRF microscopy required that the cells be cultured on collagen II-coated glass substrates. It would be very interesting, once technical challenges are overcome, to examine this organization in cells grown on soft and stiff gel substrates.

Our results and those from others suggest that receptor multimerization can act as a mechanism for mechanocoupling of TGFβ receptor signaling, and possibly for other pathways [105]. Others have shown that the solid-state presentation of ligands plays a critical role in structuring multimeric receptor clusters. For example, major histocompatibility complexes (MHC) bound to antigen presenting cells are able to structure the organization of T-cell receptors [105]. Likewise, ligands embedded in the ECM, such as TGFβ or collagen II, may be important for the structured organization of both TGFβ receptors and integrin α2β1 [65,106]. These protein complexes may create geometric constraints that structure receptor clusters and provide focal adhesions with the capability to integrate signaling between physical and biochemical cues.

This organization and its sensitivity to cytoskeletal tension have several functional implications. For example, the organized TGFβ receptors at focal adhesions would have increased access to the reservoir of tension-activated TGFβ in the ECM, especially in response to stiff substrates or mechanical loads. Furthermore, this organization might provide a mode to sequester TGFβ receptors and prevent activation of downstream signaling until the optimum threshold of physical cues is encountered. This optimum likely varies not only across cell types, but also depends on variables such as the physical properties of the microenvironment, the presence or combination of biological factors, or even the stage of cell differentiation. Although preliminary findings show this observation is conserved across multiple cell types, more work is needed to determine the extent to which this receptor organization is present in and relevant to bone and other skeletal cells. It will also be interesting to study whether this spatial organization changes during cell differentiation or in disease. During disease processes ranging from vascular disorders to osteoarthritis, the TβRII multimerization partner inappropriately switches from Alk5 to Alk1 [107]. Although we do not observe any organizational differences between Alk5 and Alk1 so far, it will be interesting to examine the spatial organization of TGFβ receptors in osteoarthritic chondrocytes, where the surrounding physical environment and the biological mechanisms are disrupted.

Downstream TGFβ effectors

Recent studies have revealed the ability of physical cues to regulate downstream components of the TGFβ pathway within the context of cell differentiation. Smad3 phosphorylation, localization, and transcriptional activity are regulated by physical cues during differentiation of chondrocytes on substrates of varying stiffness [25]. Allen et al. demonstrated that ECM stiffness is sufficient to induce Smad3 phosphorylation and nuclear translocation, as well as Sox9 and Col2α1 expression, even in the absence of exogenous TGFβ [25]. Interestingly, the combination of ECM stiffness and exogenous TGFβ synergistically induces high levels of chondrogenic gene expression. Similar findings of tension-dependent regulation of Smad3 were observed in during TGFβ-inducible epithelial mesenchymal transition [108], as well as with Smad1 in osteoinduction of MSCs grown in spread or confined configurations [109]. At a larger scale, during load-induced bone formation, mechanical load rapidly represses TGFβ signaling, leading to reduced phosphorylation and activity of downstream effectors Smad2 and Smad3 [54]. This response seems to be acutely sensitive to other factors, potentially including the type or magnitude of strain. More work is needed to elucidate the effects of static and dynamic physical cues on downstream effector Smad activity in both cartilage and bone.

In addition to the canonical TGFβ effectors like Smad3, several other effectors are known targets of both TGFβ and mechanotransduction cascades. These include myocardin-related transcription factor A (MRTF-A) [110], Yes-associated protein (YAP), and transcriptional coactivator with PDZ-binding motif (TAZ). All three can act as nuclear relays of cytoskeletal tension resulting from ECM stiffness or cell shape. For example, nuclear translocation of YAP/TAZ is dependent on Rho activity and cytoskeletal tension [111]. YAP/TAZ function is required for osteogenic differentiation of MSCs on a stiff ECM [111]. Transfer of cytoskeletal tension to the nucleus is essential for activation of YAP/TAZ signaling in response to dynamic stretch [112]. Even *in vivo,* MT1-MMP (*Mmp14*)-dependent changes in the local ECM microenvironment were required for YAP/TAZ nuclear translocation in the regulation of osteogenic differentiation [113]. YAP and TAZ are also transcriptional coregulators that can direct the nuclear localization of Smad2/3 in embryonic stem cells [114]. Whether YAP/TAZ nuclear localization contributes to the stiffness-sensitive translocation of Smad3 remains to be determined [25]. MRTF-A participates in TGFβ-inducible epithelial-myofibroblast transition, which is a mechanosensitive

process [115]. However, additional research is needed to further clarify the mechanistic role of YAP, TAZ, and MRTF-A in crosstalk between TGFβ and mechanotransduction cascades, particularly in skeletal tissues.

Molecular Model of a Physical Optimum for TGFβ signaling

These findings collectively reveal the close relationship between physical cues and TGFβ signaling, and further suggest the presence of a signaling feedback mechanism. For example, we and others have reported that the effect of substrate stiffness or cytoskeletal tension/Rho/ROCK activity on downstream TGFβ signaling is synergistic and nonlinear [25,116]. We propose a model (Figure 2) by which the combination of an optimal physical environment and exogenous TGFβ drive synergistic induction of TGFβ signaling. In a sub-optimal physical environment, TGFβ receptors are sequestered from each other at sites of adhesion. Furthermore, due to lack of cytoskeletal tension in this environment, integrins are unable to release TGFβ from its latent form. This results in basal levels of downstream TGFβ signaling. Addition of exogenous TGFβ to this microenvironment leads to an increase in TGFβ signaling away from sites of adhesion and an induction in downstream Smad effectors. In an environment with optimal physical cues – either static (e.g. ECM stiffness) or dynamic (e.g. mechanical loading) – TGFβ receptors are no longer sequestered from each other, allowing them to form a complex and initiate downstream signaling. Upon this more ideal substrate, integrins release activated TGFβ ligand from its latent form, and the ligand can bind to TGFβ receptors that are already in a primed position.

Addition of exogenous TGFβ to this physical environment results in multimerization and activation of TGFβ receptors at and away from sites of adhesion, leading to increased levels of downstream Smad activity and an ideal situation for TGFβ-inducible differentiation. Interestingly, Allen et al. demonstrated that ECM stiffness alone can induce TGFβ1 expression, which might participate in this feedback mechanism, further driving the activation of TGFβ signaling and cell differentiation in this optimal physical environment [25]. The physical cues that comprise this optimum may differ across cell and tissue types, and likely vary from development to disease. Furthermore, it is important to note that these cues most likely act through a gradient or threshold levels rather than a simple “on/off” mechanism, further adding to the complexity of this proposed mechanism.

Summary

In this review, we summarize the ability of static and dynamic physical cues to regulate TGFβ signaling from the ligand to the nuclear level in skeletal cells. We propose a mechanism that might enable cells to recognize an optimal physical environment and generate a coordinated response to the complex cues in the microenvironment, thus regulating TGFβ signaling and inducing cell differentiation. However, many questions remain regarding the cell's ability to distinguish between and integrate such cues in a manner that regulates both behavior at the cellular scale and properties at the tissue scale. It will be interesting to not only answer these questions, but also to investigate how these abilities and the gradients of cues shift between cell types or during differentiation, homeostasis, and disease progression. The continued elucidation of these mechanisms will provide essential insight into the roles that these integrated cues play in skeletal processes from development to disease.

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Figure Legends

FIGURE 1. Feedback loop integrating cytoskeletal tension and the TGFβ pathway. Cytoskeletal tension is dependent on many factors, including the material properties of the ECM (e.g. elastic modulus). In turn, cytoskeletal tension regulates the TGFβ pathway at several hierarchical levels, playing a role in TGFβ mRNA and protein expression and ligand activation; in receptor spatial organization and multimerization; in the choice among canonical Smad2/3 and non-canonical effectors; and in expression and function of lineage-specific transcription factors. These transcription factors bind to promoters of TGFβ-regulated lineage-specific ECM proteins which, through mechanisms that remain unclear, define the material properties of the ECM.

FIGURE 2. Proposed mechanism of interaction between physical cues and TGFβ in inducing skeletal cell differentiation. In a sub-optimal physical microenvironment (A, B), TGFβ receptors are segregated from each other at sites of adhesion. Due to lack of ideal cytoskeletal tension, integrins are unable to release activated TGFβ ligand from the ECM. This combination of cues leads to basal levels of downstream TGFβ signaling (A), unless exogenous TGFβ is added (B). Upon addition of TGFβ, TGFβ receptors away from sites of adhesions are able to bind the ligand and initiate downstream signaling (B). In an optimal physical microenvironment (C, D), the physical separation of TGFβ receptors at sites of adhesion is released. The receptors are able to bind active ligand that was released by integrin interactions with LAP (C). Addition of TGFβ to the optimal physical microenvironment leads to a synergistic induction of downstream TGFβ signaling (D).

TABLE 1. Effects of physical cues on TGFβ signaling

Level of TGFβ pathway			
Molecule, behavior	*Physical cue*	*Cell/tissue type*	*Reference*
mRNA and protein expression			
TGFβ1 mRNA	Substrate stiffness	Chondrocytes	Allen et al. [25]
TGFβ1 protein	Substrate stiffness	Chondrocytes	Allen et al. [25]
TGFβ mRNA	Mechanical load	Bone tissue	Raab-Cullen et al. [82]
TGFβ1 protein	Shear stress	Osteoblasts	Sakai et al. [83]
TGFβ1,3 mRNA	Cyclic compression	MSCs, chondrogenic	Li et al. [84]
TGFβ1,3 protein	Cyclic compression	MSCs, chondrogenic	Li et al. [84]
TGFβ1 protein	Topography	Osteoblasts	Lohman et al. [85]
Ligand			
TGFβ1 ligand, activation	Cytoskeletal tension, substrate stiffness	Myofibroblasts	Wipff et al. [13]
TGFβ1 ligand, activation	Shear stress	Cartilage tissue	Albro et al. [92]
TGFβ ligand, activation	Mechanical stretch	Tendon fibroblasts	Skutek et al. [94]
TGFβ ligand, activation	Tensile load, shear stress	Tendon, tenocytes	Maeda et al. [93]
Receptor			
TGFβ receptor, organization	Cytoskeletal tension, ROCK activity	Chondrocytes	Rys, DuFort, et al. [65]
Effectors			
Smad3, nuclear translocation	Substrate stiffness	Chondrocytes	Allen et al. [25]
Smad3, phosphorylation	Substrate stiffness	Chondrocytes	Allen et al. [25]
Smad2/3, phosphorylation	Mechanical load	Bone tissue	Nguyen et al. [54]
Smad1, nuclear translocation	Cell shape, Cytoskeletal tension, RhoA/ROCK activity	MSCs, osteogenic	Wang et al. [109]
Smad1, phosphorylation	Cell shape, Cytoskeletal tension, RhoA/ROCK activity	MSCs, osteogenic	Wang et al. [109]
YAP/TAZ, nuclear translocation	Substrate stiffness, Rho activity	MSCs, osteogenic	Dupont et al. [111]
YAP/TAZ, nuclear translocation	Dynamic stretch, strain transfer	MSCs	Driscoll et al. [112]
Skeletal cell differentiation			
Sox 9, Collagen II	Substrate stiffness	Chondrocytes	Allen et al. [25]
Alkaline phosphatase	Cell shape, Cytoskeletal tension, RhoA activity	MSCs, osteogenic	McBeath et al. [46]
Collagen II	Substrate stiffness	Chondrocytes	Park et al. [50]

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FIGURE 1

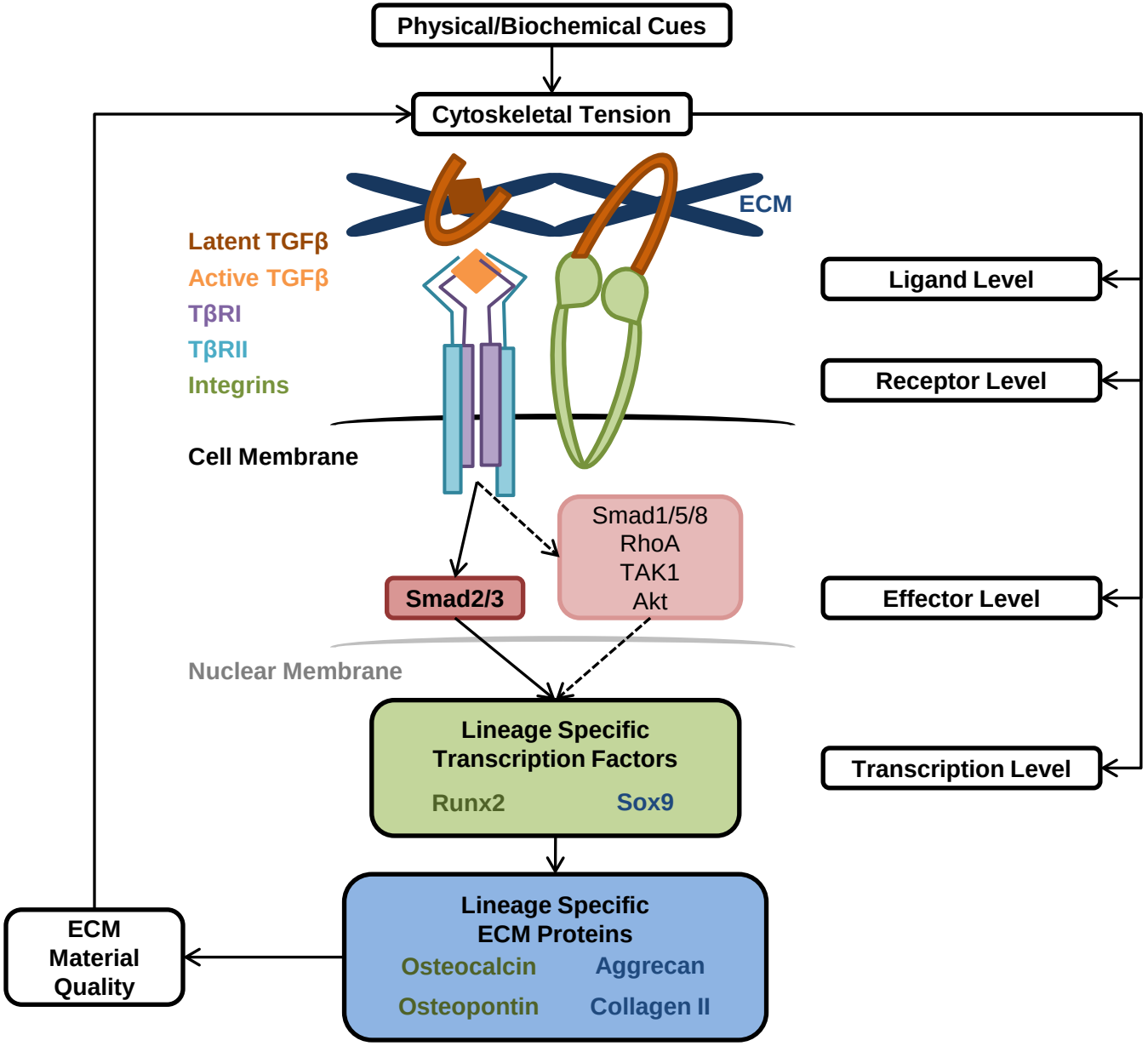
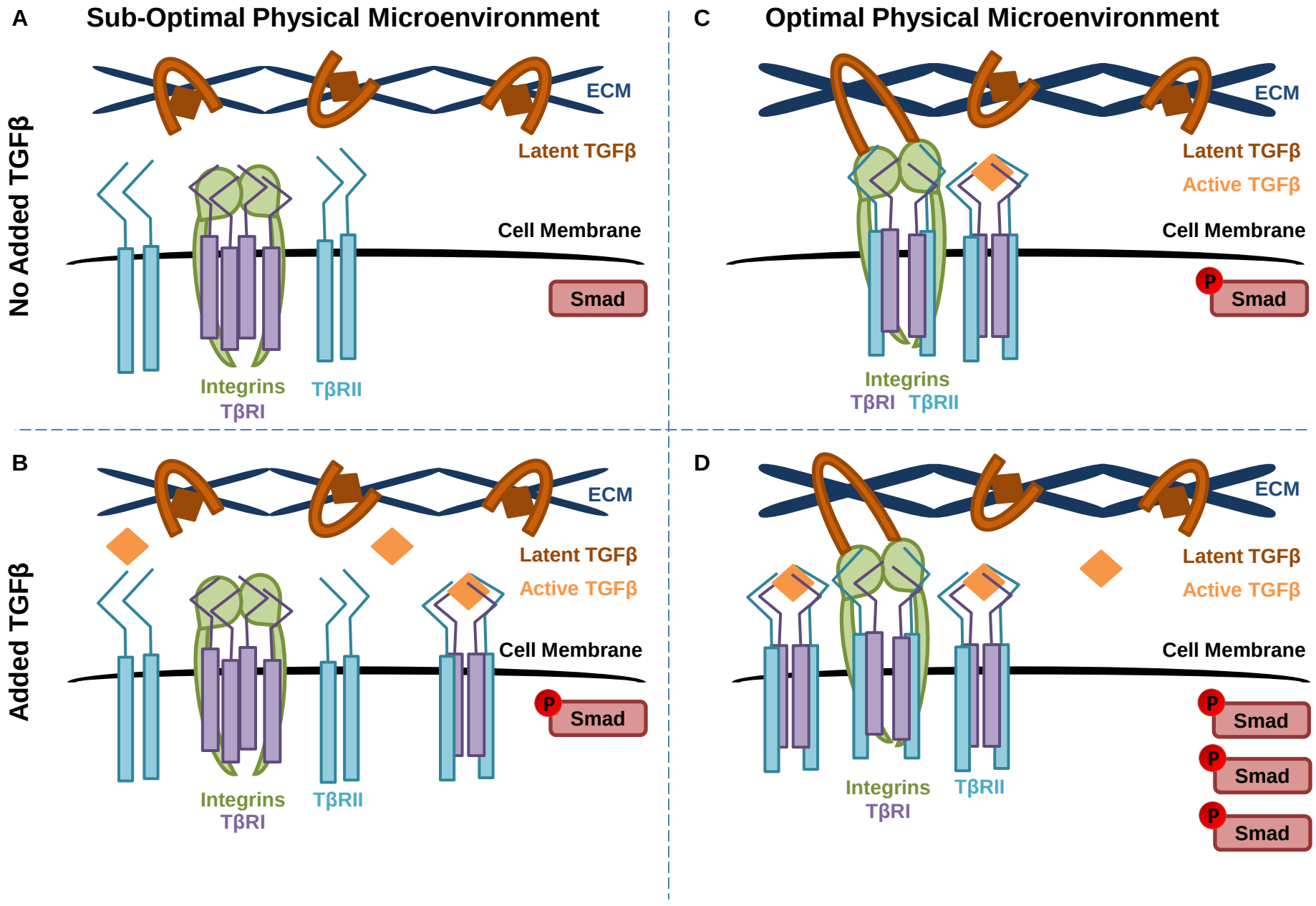

Physical/Biochemical Cues
Cytoskeletal Tension
ECM
Latent TGFβ
Active TGFβ
TβRI
TβRII
Integrins
Cell Membrane
Ligand Level
Receptor Level
Smad1/5/8
RhoA
TAK1
Akt
Smad2/3
Effector Level
Nuclear Membrane
Lineage Specific
Transcription Factors
Runx2
Sox9
Transcription Level
Lineage Specific
ECM Proteins
Osteocalcin
Aggrecan
Osteopontin
Collagen II
ECM
Material
Quality

FIGURE 2


A
Sub-Optimal Physical Microenvironment
No Added TGFβ
ECM
Latent TGFβ
Cell Membrane
Smad
Integrins
TβRI
TβRII
C
Optimal Physical Microenvironment
ECM
Latent TGFβ
Active TGFβ
Cell Membrane
P
Smad
Integrins
TβRI
TβRII
B
Added TGFβ
ECM
Latent TGFβ
Active TGFβ
Cell Membrane
P
Smad
Integrins
TβRI
TβRII
D
ECM
Latent TGFβ
Active TGFβ
Cell Membrane
P
Smad
P
Smad
P
Smad
Integrins
TβRI
TβRII