Published in final edited form as:

Ann N Y Acad Sci. 2017 December ; 1409(1): 18–32. doi:10.1111/nyas.13427.

Mechanobiology of limb musculoskeletal development

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Abstract

While there has been considerable progress in identifying molecular regulators of musculoskeletal development, the role of physical forces in regulating induction, differentiation, and patterning events is less well understood. Here, we highlight recent findings in this area, focusing primarily on model systems that test the mechanical regulation of skeletal and tendon development in the limb. We also discuss a few of the key signaling pathways and mechanisms that have been implicated in mechanotransduction and highlight current gaps in knowledge and opportunities for further research in the field.

Keywords

musculoskeletal; tendon; mechanics; development; mouse

Introduction

The development of the musculoskeletal system is a carefully orchestrated process that gives rise to cartilage, bone, tendon, and muscle. While it has been established that key events in tissue induction, differentiation, migration, and patterning are driven by soluble morphogens acting on cells (both spatially and temporally), there is now growing appreciation that physical force is also an important driver of developmental processes. This field of study, broadly termed mechanobiology, focuses on the role of mechanical forces in regulating biologic phenomena. Force inputs can be generated within the cell via cellular interactions with the extracellular matrix or across cell–cell contacts. These physical forces can then directly affect biological activities, such as proliferation, apoptosis, differentiation, and migration. The coupling of force inputs to biological outcomes is mediated by highly conserved signaling pathways that are activated by mechanical forces.

Since the main function of musculoskeletal tissues is to bear and transmit loads, it is unsurprising that these tissues are highly mechanoresponsive, and mechanical forces have been implicated in every aspect of their biology, from development to homeostasis to pathology. While there is considerable interest in these topics, the majority of studies rely on experiments carried out on isolated cells under artificial conditions. These studies provide

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Competing interests

The authors declare no competing interests.

important information on signal transduction (since the application of forces can be carefully controlled, timed, and measured); however, the gap between *in vitro* observations and *in vivo* phenomena must still be bridged. Since there are numerous excellent reviews focusing on mechanotransduction within *in vitro* contexts,^{1–3} we focus primarily on *in vivo* model systems that test mechanical regulation of musculoskeletal development, with particular emphasis on skeletal (cartilage, joint, and eminence/enthesis) and tendon tissues in the limb, since there has been a significant body of work in this area. We will also discuss a few of the key signaling pathways that have been implicated in mechanotransduction and highlight current gaps in knowledge and opportunities for further research in the field.

***In vivo* animal models for mechanobiology**

The use of genetic and chemical manipulations in animal model systems have advanced our understanding of how mechanical forces regulate skeletal development, homeostasis, and healing.^{4–6} While a wide range of animal models have been used, we focus on the chick and mouse systems owing to the greater similarity of musculoskeletal structures and development with humans (in contrast to invertebrates and lower-order vertebrates). The choice of chick versus mouse depends on specific questions of interest, since each system confers distinct advantages. The chick is an attractive model for mechanotransduction experiments because chick development occurs externally within an egg. The embryo is therefore easily manipulated, and direct mechanical perturbations can be applied or measured during developmental stages. Immobilization can be induced by treatment with small molecules or by surgical removal of muscles before their migration into the limb. The chick is the preferred animal for the use of neuromuscular modulators during developmental stages, as pharmaceutical agents can be easily delivered *in ovo*. Placental transfer in mammals prevents transport of neuromuscular agents owing to their ionic nature, which limits the use of available neuromuscular agents during mammalian embryogenesis.⁷ A number of neuromuscular modulators have been broadly used to either induce or prevent skeletal loading *in ovo* (summarized in Table 1). Decamethonium induces motor endplate depolarization resulting in rigid paralysis, while pancuronium, a nicotinic acetylcholine antagonist, can be used to induce flaccid paralysis.^{8, 9} Other widely used molecules that induce flaccid paralysis include botulinum toxin,¹⁰ succinylcholine,¹¹ and tubocurarine.¹² Lastly, surgical and grafting techniques can also be extremely powerful for studying development outside of native environments and can be paired with small molecules to further elucidate region- or tissue-specific mechanisms governing loading-dependent morphogenesis.

In addition to the chick, the mouse has also emerged as a powerful model for studying the role of physical forces during development, since genetic manipulation is limited in the chick. The mouse is the most widely used mammalian model organism, and there are a wide array of molecular tools for targeting and labeling specific cells and tissues. Although gestational times for chick and mouse embryos are both ~3 weeks, the development of limbs and musculoskeletal structures occurs on very different days. Therefore, to simplify the nomenclature in this review, embryonic staging days are only provided for mouse. The majority of mechanotransduction studies in mouse rely on immobilization, and there are several muscle-deficient mutants that confer immobilization phenotypes by arresting either

muscle development or contraction (summarized in Table 1). In the limb, muscle progenitors migrate from the somites into the limb during early limb bud development. The progenitors then differentiate and fuse to form myofibers, and active muscle forces are initiated at ~E13.^{13, 14} The most common muscle-deficient mutants include those that target muscle progenitor migration into the limbs, muscle differentiation, or muscle activity. In *Pax3* mutants (such as *Spotch* or *Spotch-delayed*), migration of muscle progenitors is abolished, and developing limbs are therefore “muscle-less.”¹⁵ *Spotch* (*Sp*) mutants are embryonic lethal by ~E14.5, which prevents the study of skeletal growth and remodeling past this time point. However, *Spotch delayed* (*Sp^d*) mutant embryos display the same muscle-less phenotype but survive until the end of embryogenesis at E18.5, since the disruption of *Pax3* is less severe.¹⁶ Mutations targeting muscle differentiation include disruptions to muscle-regulatory transcription factors (MRFs: *MyoD*, *Myf5*, *Mrf4* and *myogenin*). *MyoD* and *MYF5* were the first transcriptional regulators identified that were necessary for the formation of skeletal muscle.^{17, 18} *MyoD*^{-/-} *Myf5*^{-/-} double-null mutants show a muscle-less phenotype, due to arrest of skeletal muscle differentiation.¹⁹ A less severe deletion of these genes (such as *MyoD*^{-/-} *Myf5*^{+/-} embryos) results in a reduced muscle mass phenotype, providing a model for stress dosing.²⁰ Finally, the role of active muscle contraction in development can be determined using mutants such as the muscular dysgenesis mutant (*mdg*). The *mdg* embryos carry a loss-of-function mutation in voltage-gated Ca²⁺ channels preventing skeletal muscle depolarization and contraction.²¹ Muscle progenitor migration, differentiation, and patterning are normal in *mdg* mutants; however, excitation–contraction coupling is lost, resulting in paralyzed muscles. In the absence of active muscle loading, the muscles themselves display an abnormal ultrastructural appearance beginning at E14,²² suggesting that muscle activity initiates before E14 and is also required for maintenance of muscle. Collectively, these muscle mutants have been used to study the development of cartilage, bone, joint, and tendon tissues. While the advantage of the mouse system is largely due to the ability to control genetics, none of the muscle mutants discussed can be used for postnatal experimentation owing to lethality at birth or embryonic stages. For postnatal stages, the application of chemical agents, such as botulinum toxin, or direct denervation via surgery is frequently used.

The role of mechanical forces in musculoskeletal development

Skeletal development is initiated early in the limb bud, beginning with induction of *Sox9*-expressing pre-chondrogenic progenitors from the lateral plate mesoderm at E10.0. The progenitor cells that will form the skeletal elements of the stylopod (arm/thigh), zeugopod (forearm/shin), and autopod (hand/foot) are induced progressively in the proximal to distal direction.⁶ Chondrogenic differentiation of the skeletal anlagen is defined by expression of cartilage genes *Col2a1* and *Acan*. Endochondral ossification of the cartilage template is driven by the transcription factors RUNX2 and OSX; while *Runx2* is expressed as early as E11.5,²³ *Osx* is not expressed until E13.5, followed by the first appearance of ossified tissue at E14.5.⁶ As the primary limb skeleton is forming, muscle is also developing in parallel, and much of the early events regulating their development appear to be tissue autonomous. Muscle progenitors enter the limb from somites, a process that is complete by E10.5; muscle differentiation is then initiated by expression of the MRFs.²⁴ Integration of muscle with the

skeleton does not occur until ~ E12.5, when *Scleraxis* (*Scx*)-expressing tendon progenitors first connect the two tissues; thus, muscle mediated forces cannot directly act on the skeleton before this time. As the molecular programs that guide skeletal development continue to be elucidated, it is becoming clear that mechanical signals are also involved in their differentiation and patterning.

Skeletal and bone development

Skeletogenesis in the limb can be mechanically regulated via two basic schemes: *growth-generated strains* and *muscle loading* (Fig. 1).^{25, 26} Although the first muscle fibers appear in mice at E11, innervation of primary muscle fibers occurs later,¹⁴ after the initial events that prime the skeletal developmental program.¹³ Thus, growth-generated strains may be the dominant mechanical signals driving early skeletal development. During early stages (E11.5–12.5), a fibroblastic barrier develops between the skeletal primordia and the surrounding mesenchyme, ultimately forming a mature perichondrium by E14.^{27, 28} A *perichondral constraint model* was initially proposed by Wolpert whereby radial growth of the developing skeletal structure is constrained by the perichondrium, which leads to preferential growth along the long axis (Fig. 1A).²⁹ This idea is supported by studies in chicks where removal of the perichondrium positively regulates regional cartilage proliferation.³⁰ More recently, it was also demonstrated that mechanical stress applied to perichondrial cells derived from the developing limb can induce secretion of molecular cues that affect skeletal growth. Embryonic chick tibiotarsi stripped of their perichondria and treated with conditioned media from perichondrial cells grown on substrates with varying stiffness were found to exhibit growth proportional ($P = 0.01$) to substrate stiffness.³¹ Although these studies are suggestive, the extent to which perichondrial constraint mechanically directs skeletal growth remains an open question. However, the perichondrium is likely an important signaling center able to regulate chondrocyte maturation and hypertrophy, as shown in chick *ex vivo* cultures.³²

Following innervation of myofibers, muscle contraction imposes directional stresses and strains on the developing limb-bud skeleton (Fig. 1B), and a substantial body of literature suggests that cyclic muscle loading is necessary for proper skeletal morphogenesis. Immobilization studies using neuromuscular modulators during embryogenesis showed that paralysis results in skeletal malformations in chicks.^{26, 33–35} Skeletal defects were also observed in a number of mouse muscle mutants. For example, long bone growth is diminished in *MyoD*^{-/-}; *Myf5*^{nlacZ/nlacZ} muscle-less mutants, while the shape of the humeral tuberosity is altered in *Pax3*^{Sp/Sp} mutants.²⁰ In a reduced muscle-mass model, *MyoD*^{-/-}; *Myf5*^{nlacZ/+} embryos display an intermediate phenotype indicating a possible stress-dosing effect.²⁰ Interestingly, phenotypes varied by anatomic location, suggesting potential region-specific regulatory mechanisms in which skeletal development is variably affected by muscle loading.^{20, 36} One possible explanation for this observation is the role of passive forces in skeletogenesis. Finite element analysis of mouse humerus and femur showed that loss of muscle contraction alone was insufficient to explain defects in bone formation. Passive forces incurred through fetal movement in the amnion following maternal or littermate external forces have been suggested as a possible explanation for region-specific differences in muscle loading response.³⁷

Joint development

As early as 1901, the rhythmic contraction of muscle fibers was thought to be a potential regulator of joint formation.³⁸ Early evidence was uncovered in calves with abnormal spinal cords or limb muscle; in these animals, joints were ankylosed, suggesting a requirement for physical forces in the development of joint structures.³⁸ During embryogenesis, synovial joint formation occurs in two stages: joint specification followed by joint cavitation (Fig. 1C). Specification of joint cells begins at E13.5 and is characterized by loss of chondrogenic markers (SOX9 and COL2a1) at the site of the presumptive joint (termed the interzone).^{39–41} Interzone cells adopt a flattened morphology and turn on joint markers such as GDF5, WNT4, and WNT9a.^{39–41} Following joint specification, cavitation results in the separation and subsequent formation of two distinct articular surfaces, synovium, and surrounding joint structures (such as the assorted ligaments that connect bone to bone).⁴² This transition, once thought to be solely controlled by molecular signaling pathways, is in fact partly regulated by muscle contraction. A number of studies using chick paralysis models revealed that muscle activity is necessary for joint specification. In the absence of muscle forces, chondrogenic genes are maintained, and the interzone is not induced.^{33–35, 43–46} Similarly, in the absence of muscle (such as in *Pax3^{Spd/Spd}* muscle-less embryos), *Sox9* and *Col2a1* expression is maintained beyond the initial joint specification stage, resulting in loss of joints.⁴⁷ Interestingly, similar to region specificity in bone development, not all joints were similarly affected; while some joints were lost, others developed normally. The presence or absence of joints in response to loss of muscle appears to be associated with canonical Wnt signaling via β -catenin. Maintenance of Wnt signaling was observed in successful joint formation, while loss of β -catenin expression was only observed in affected joints in *Sp^d* mice.⁴⁷ This also suggests that different joints may experience mechanical loading differently or that there may be muscle-independent loading mechanisms. One example is acetabular hip development, where asymmetric prenatal movements of the femoral head lead to deformity of the hip joint, highlighting the importance of passive movement along with muscle loading in joint development.⁴⁸ Despite these intriguing findings, the mechanisms by which chondrocytes sense the mechanical signals imparted by the contracting muscles and translate these signals to activate Wnt signaling and interzone markers remain unknown.

Bone eminence development

The bone eminences are protrusions decorating the primary long bone structures and are areas of integration between tendons and the skeleton. These superstructures function to efficiently distribute loads from tendon to bone while minimizing stresses at the tendon–bone interface (termed the enthesis).⁴⁹ Recent studies have added considerable insight into the development of these secondary structures, which were once thought to be derived from the primary skeleton. In fact, the eminences are derived from an independent population of bipotent SOX9⁺/SCX⁺ eminence progenitors, which are first induced at E12.5.^{50, 51} Induction is followed by cartilage differentiation, and formation of bone occurs via endochondral ossification.^{50, 52} Although the bone eminences serve a key mechanical function, the loss of mechanical forces does not affect eminence development until relatively late stages. Experiments using *Sp^d* and *mdg* mutants revealed that muscle and muscle contraction are not required for the initiation or cartilage differentiation of eminences, but

are necessary for subsequent maintenance and growth. In the absence of muscle loading, chondrocyte proliferation is reduced, and the eminences are lost by E18.5.⁵²

Bone enthesis development

Since tendon and bone differ dramatically in material properties, the formation of stress concentrations at the interface can lead to detachment of tendons.⁵³ The mature enthesis therefore functions to further reduce stress concentrations at the interface by providing a transitional gradient of tendon, fibrocartilage, mineralized cartilage, and bone. At birth, the tendon-to-bone enthesis exists as an abrupt transition from tendinous tissue to bone.⁵⁴ Although the cells that give rise to the enthesis are specified during embryonic stages (at E14.5), differentiation and maturation of the tissue occurs at postnatal stages and depends on muscle forces.^{51, 55} While initiation of the enthesis fibrocartilage is independent of muscle contraction, paralysis experiments using botulinum toxin administered after birth showed that fibrocartilage differentiation and maintenance, as well as mineralization of the tissue gradient, require active muscle loading.^{26, 51, 56–59}

Tendon development

Compared with skeletal and muscle formation, the developmental sequence of tendon formation was established only recently. Although a few markers have been used in an attempt to identify and follow early tendon progenitors, these markers were either non-specific to tendon (such as Tenascin or COL1a1) or were expressed only by differentiated tendon cells (such as TNMD), and thus did not fully capture the earliest events of tendon development. Identification of the transcription factor *Scx* as a useful marker for early tendon progenitors first allowed detailed analysis of tendon development and the generation of tendon-specific mouse tools.^{60–62} This section will discuss tendon development in the context of mechanical regulation; however, an in-depth discussion of cell and molecular regulators of tendon development can be found in several recent reviews.^{63–65}

In the limb bud, *Scx* is first expressed by limb mesoderm at E10.5, following induction of *Sox9*-expressing pre-chondrogenic progenitors at E10.0.⁶¹ At early stages (E10.5–11.5), the domains of *Scx* expression are closely associated with those of muscle, although *Scx*⁺ and *PAX3*⁺ progenitors are completely distinct throughout all developmental stages.⁶⁵ Despite early induction of *Scx*, a characteristic tendon pattern is not apparent until E12.5,^{61, 62} when aligned progenitors can be observed within all segments of the limb, connecting muscle to the skeleton.^{65–67} Generally, the stages of limb tendon development can be defined as induction (E10.5–E12.5), differentiation (E13.5), and growth (E14.5 onward). Recent studies established a new understanding of limb tendon development, in which tendons in the autopod are regulated by a separate developmental program relative to tendons in the zeugopod or stylopod.^{66, 68, 69} Since autopod tendon development is unique to this segment, we will discuss tendon development in the context of the zeugopod, which is more representative of other long tendons within the body. In this new conceptual model, long tendons develop via a two-phase process whereby (1) muscle first attaches to the cartilage skeleton via a short tendon segment at E12.5, followed by (2) elongation of the tendon from E13.5 onward to generate a long, linear structure.

Although the first phase of tendon induction is independent of muscle, regulation of the second phase (tendon elongation) is likely due to physical signals imparted by both the muscles and the skeleton. Using *Pax3^{Spd/Spd}* muscle-less mice, it was established that the short range tendon anlagen is induced at E12.5 in the absence of muscle but subsequently lost at E13.5.⁶⁶ Interestingly, the requirement for muscle in the elongation of tendon structures did not depend on muscle activity, since tendon elongation appears normal in paralyzed *mdg* limbs.⁶⁶ Since successful force transmission depends on optimal tension and proportional length between muscle and tendon relative to the skeleton, one potential regulator of longitudinal tendon growth could be the skeleton itself. This phase of tendon elongation may therefore be indirectly coupled to skeletal growth via attachment to muscle, such that the extent of skeletal growth may impart a stretching signal to tendons that dictates tendon growth. In the absence of muscle, the tendon segment is not integrated with the skeleton, which may lead to degeneration of the tendon in the absence of tensional force. Although conceptually intriguing, specific experiments directly testing the mechanical role of skeletal growth in tendon elongation have not yet been carried out.

There is, however, some indirect evidence that tension may be important for tendon elongation, based on the unique developmental program governing a specific set of anatomical muscles, the superficial flexors (flexor digitorum superficialis (FDS) muscles). In the mouse, the FDS muscles initially form as three individual muscles in the autopod, each attached to a short-range tendon fragment located at the metacarpal phalangeal (MCP) joint of digits 2–4 (E14.5).^{67, 70} The FDS muscles subsequently translocate out of the autopod and cross the wrist into the zeugopod such that, by E16.5, the muscles are completely located within the zeugopod. Interestingly, as the muscles move out of the autopod (E14.5–16.5),⁶⁷ the three short tendons elongate dramatically to form three long tendons that traverse the entire distance from the MCP joint in the autopod to their muscle origins in the zeugopod. In this case, the final length of FDS tendons is not dictated by skeletal growth, but rather the extent of muscle translocation. In cases where muscle translocation is arrested, longitudinal growth of the FDS tendons is similarly arrested. Thus the actively moving muscle likely imparts a tensional force on the tendons, akin perhaps to the force imparted by skeletal growth that is sensed by limb tendons in general.

While overall tendon elongation is not affected in *mdg* limbs, tendons are noticeably thinner by E16.5,⁶⁷ suggesting a role for muscle contraction in regulating lateral tendon growth or robustness. This is supported by the phenotype of the autopod tendons in muscle-less limbs (which are capable of forming in the absence of muscle), as they are also dramatically thinner in size. Whether lateral growth at these stages depends on cell proliferation, new cell recruitment, or deposition of extracellular matrix has also not yet been determined. To date, the molecular signals that mediate tenocyte responses to physical stimuli have not been fully identified, but a few have been implicated as mechanosensitive on the basis of *in vitro* tissue culture experiments and a few *in vivo* experiments.⁶³ These include transcription factors such as *Scx* and signaling pathways, including TGF- β and FGF.^{71, 72}

Molecular regulators of mechanobiology in development

The regulation of musculoskeletal development by mechanical forces depends on successful detection of mechanical signals by the cell and subsequent activation of biological processes. While it is clear from whole-organism studies that mechanical loading is necessary for some aspects of musculoskeletal morphogenesis, specific mechanotransduction pathways can be difficult to test *in vivo*, although there is evidence that disruption of known pathways results in arrest or abnormal development.^{59, 72, 73} In general, detailed understanding of mechanobiology pathways relies heavily on *in vitro* culture systems that allow for direct perturbations and measurements of forces on the cellular level. We therefore discuss three mechanisms that may potentially regulate the transduction of mechanical signals to cells in the embryo: (1) cell–extracellular matrix (ECM) transduction, (2) cell–cell interactions, and (3) primary cilium sensing.

Cell–ECM transduction

The cellular microenvironment consists of biophysical, chemical, and cellular cues that are responsible for cell homeostasis. Of these components, the ECM plays a critical role in providing cellular scaffolding and sequestration of bioactive factors, thus influencing cell shape, proliferation, migration, and differentiation.^{2, 74–76} The ECM is constantly remodeled as cells deposit and degrade ECM in response to microenvironmental cues. During development and wound repair, ECM dynamics are dramatically accelerated to allow for restructuring of tissue architecture. Notably, it was shown that the mechanical properties of the ECM (such as stiffness/elasticity) are themselves potential regulators of stem cell differentiation such that cells favor lineages that tension match the elasticity of the underlying substrate (e.g., stem cells on soft substrates favor neurogenic lineages, while stem cells on rigid substrates favor osteogenic lineages).⁷⁶ These data suggest that cell–ECM mechanical signals may maintain healthy cell and tissue-specific phenotypes under homeostasis; however, if the homeostatic microenvironment is disrupted, newly deposited ECM can also guide progenitor cells back toward a healthy tissue architecture or toward an aberrant pathological state via positive or negative feedback loops.

Cell–ECM transduction initiates with transmembrane integrins binding to ECM proteins, which then transmits force across the plasma membrane of the cell to the focal adhesion complex (FAC) (Fig. 2). One example highlighting the importance of ECM molecules during embryogenesis was described by George *et al.* in 1993, in which fibronectin gene null mutants display defects in mesodermal, vascular, and neural tube development.⁷⁷ It has also been shown that, during somitic development, mesodermal compaction depends on integrin–ECM interactions.⁷⁸ In addition, AER formation during limb-bud outgrowth depends on the transition of epithelial cells from squamous to columnar, which is abolished in double null integrins $\alpha_3^{-/-} \alpha_6^{-/-}$ mutants, further illustrating the necessity of proper cell–ECM signaling.⁷⁹ Multiple anchoring proteins are involved in coordinating the binding of cytoskeletal actin to β -integrin subunits and are incorporated within the FAC. One key protein, talin, has emerged as an anchoring protein necessary for actin–integrin linkage. In a remarkable study, “laser tweezers” were used to establish the requirement for talin1 in 2-pN binding of actin to $\alpha_v\beta_3$ integrins.⁸⁰ In talin-depleted mutants, integrin binding does not

lead to actin linkage.⁸¹ Additional studies demonstrated that talin not only functions to form actin–integrin linkage, but also maintains and strengthens the FAC. In particular, vinculin association with talin helps to cluster actin–integrin links to further increase focal adhesion kinase (FAK) strength in response to substrate tension.⁸² Mechanical stretching of talin itself can reveal cryptic vinculin-binding sites, as was elegantly demonstrated using “magnetic tweezers.”⁸³ Cell–ECM interaction is also required for maintenance and development of the myotendinous junction (MTJ), and expression of talin-2 and integrins (such as ITGA7) is necessary for proper MTJ function.^{84, 85}

Following FAC formation, multiple adaptor proteins localize to the FAC, forming the adhesome, which can activate a number of signaling molecules, including G proteins, phosphatases, kinases, and other regulators (Fig. 2).⁸² The Rho family of GTPases—Rho, Rac, and Cdc42—are known regulators of actin dynamics and are responsible for the generation of cytoskeletal forces. Importantly, Rho GTPases play pivotal roles in cell polarity, migration, and cell shape.⁸⁶ Recently, RhoA was shown to regulate stem cell differentiation along osteogenic lineages by controlling cell shape and actin cytoskeletal tension.⁸⁷ Rho activation by guanine exchange factors (GEFs) leads to phosphorylation of Rho-associated protein kinase (ROCK). Downstream effectors of ROCK activation include filament stabilization and myosin light chain kinase (MLCK)-mediated actinomyosin contraction. Interestingly, actinomyosin contraction is necessary for BMP-2-mediated osteogenic differentiation of human mesenchymal stem cells, indicating that canonical growth factor signaling can be modulated by cell–ECM mechanotransduction.⁸⁸ Similarly, activation of Rac requires activation of the Rac GEF, DOCK180-ELMO.^{82, 89} The FAC adapter proteins p130CAS and paxillin (which are also mechanosensitive) in turn activate DOCK180-ELMO.^{90, 91} Recently, the LIM protein zyxin has emerged as an “ultrafast” mechanosensor response for actin stress fiber maintenance and repair.^{92, 93}

Analysis of the FAC indicates that the adhesome consists of roughly 160 distinct components, each acting in coordination with one another at the FAC. Thus, there likely exist additional mechanisms by which mechanical stimuli are sensed or are coordinated with other distinct signaling pathways. However, it is clear that cell–ECM-mediated mechanical cues lead to the formation of a highly diverse adhesome that is likely to have far-reaching implications on cellular phenotypes. Indeed, while much of the focus on mechanotransduction is at the cellular membrane, FAK-mediated tension generated within the cytoskeleton is now thought to shape nuclear architecture via actin–nuclear laminin couplings, modulating chromatin organization and expression.^{94–96} Recent work also demonstrated that mechanotransductive cues from the ECM act on the downstream regulators Yes-associated protein (YAP) and transcriptional co-activator with PDZ binding motif (TAZ) of the Hippo signaling pathway.^{97, 98} The Hippo pathway is driven by activation of the upstream regulator mammalian-STE20 like protein kinase 1 (MST1) and MST2, leading to phosphorylation of the large tumor suppressor homologue 1 (LATS1) and LATS2, which phosphorylate YAP and TAZ, resulting in cytoplasmic degradation.⁹⁹ Inactivation of this pathway leads to nuclear translocation of YAP/TAZ and binding to the TEA domain (TEAD) family members, inducing proliferation. While the vast majority of current research is focused on the role of YAP and TAZ in cancer, Hippo signaling was first identified on the basis of their activity in regulating organ size during development.^{100, 101}

The coupling of cell–ECM signaling to Hippo signaling provides a potential mechanism to understand how mechanical cues may help to direct cellular processes (such as proliferation, differentiation, or apoptosis) to guide developmental events. In a seminal paper, Dupont *et al.*, found that YAP/TAZ nuclear shuttling was highly controlled by the activity of Rho GTPases (Fig. 2).¹⁰² Rho signaling also inhibits LATS activity and is required to maintain pluripotency of human embryonic stem cells.¹⁰³ While the mechanisms by which F-actin polymerization can lead to YAP activation are unclear, the best-known model suggests that F-actin competes with YAP for the binding site on the inhibitory protein angiomotin (AMOT), thereby increasing the abundance of active YAP.¹⁰⁴ While there is still much to be investigated, it is clear that the role of cell–ECM–mediated stress, both at the FAC–adhesome and through cytoskeletal dynamics, can play a pivotal role in shaping basic cell biology.

Cell–cell interactions

During early developmental stages when the contribution of ECM may be minimal, cell–cell interactions act to transmit mechanical signals across cell populations. Cadherins are the primary cell–cell adhesion proteins that transmit intracellular-generated traction forces from one cell to another. These forces are primarily involved in maintaining tissue integrity, but also serve to transmit mechanical cues similar to cell–ECM transduction. Cadherins are transmembrane proteins that join cells at their extracellular domains (Fig. 3). Although the organization of the intracellular components remains subject to considerable debate, one model proposes an adaptor protein complex consisting of p120, β -catenin, and α -catenin associated with the intracellular domain of the cadherin linked to the cytoskeleton.¹⁰⁵ Myosin-driven contractility at the cadherin complex induces conformational changes in α -catenin, exposing vinculin-binding sites, illustrating the mechanosensory capacity of cadherins.^{106, 107} Furthermore, recent work suggests that Rho GTPase activity is necessary for the establishment of cadherin-dependent cell–cell adhesions.¹⁰⁸ Overall, the intracellular signaling pathways triggered by cell–cell mechanical cues shows a high degree of overlap with those described in cell–ECM interactions (Fig. 3).

While the mechanisms of cadherin signaling continue to be elucidated, it is clear that cadherins play a large role in planar cell polarity, morphogenesis, and early cell migration in the developing embryo and can activate key pathways, such as Wnt or receptor tyrosine kinase pathways.^{109–111} Although there are several cadherins, N-cadherin is of particular interest to the musculoskeletal community for its role in chondrogenesis. In the developing limb bud, N-cadherin expression is temporally and spatially associated with condensation and is required for chondrogenesis.¹¹² These discoveries have prompted tissue-engineering efforts aimed at manipulating cadherin signaling to generate cartilage *in vitro*.^{113–115}

Primary cilium sensing

Primary (or non-motile) cilia are solitary organelles found on all mammalian cells. Although primary cilia were once thought to be purely vestigial, it is now appreciated that these important organelles play critical functions during development and present another route by which cells sense mechanical signals.¹¹⁶ The primary cilium is composed of a cytoskeleton scaffold called an axoneme, which provides binding sites for motor proteins to facilitate

shuttling of proteins up and down the cilium. At the cilium base are basal body proteins (BBPs) that form the BBsome, which is required for formation and maintenance of the cilium.¹¹⁷ Intraflagellar transport proteins bound to dynein and kinesin motor proteins shuttle intracellular cargo up and down the cilia (Fig. 4). Most cells assemble only one cilium, which is resorbed during mitosis and is reassembled following exit from the cell cycle.

Mutations in BBPs or other cilia components are associated with a host of human congenital diseases, collectively known as ciliopathies.^{116, 118, 119} Phenotypes associated with ciliopathies are extremely wide ranging and variable and have not been fully defined. Of the known ciliopathies, one of the best studied is autosomal dominant polycystic kidney disease (ADPKD), which involves mutations in the genes *PKD1* and *PKD2*.¹²⁰ In ADPKD, renal cells are insensitive to fluid flow despite the presence of primary cilia.¹²¹ This disease model was one of the earliest to establish a function for primary cilia in mechanosensation. However, a number of other examples have emerged in recent years, including the establishment of left–right asymmetry and cardiac looping during embryogenesis.¹²²

The downstream pathways linked to primary cilia have been subject to some debate, with the exception of hedgehog signaling. It is well established that hedgehog signaling is coordinated by primary cilia, and gene mutations of primary cilia components frequently recapitulate phenotypes associated with dysfunctional hedgehog signaling.^{123–125} Examples in musculoskeletal development include endochondral ossification in long bone growth (regulated by *Ihh*) and digit patterning (regulated by *Shh*). Conditional mutants for cilia components IFT88 or KIF3a in limb mesenchyme result in phenotypes associated with abnormal hedgehog signaling, including shortened limb skeletons and polydactyly.^{126, 127} Other pathways, including Wnt, PDGFR α , TGF- β , and Ca²⁺ signaling have also been implicated in primary cilia signaling; however, additional studies are required to establish whether these pathways are directly activated by cilia.^{117, 128–130}

The mechanical signals that may be sensed by primary cilia include fluid flow, osmotic pressures, and tensile strains.^{131–133} These signals are present in a number of musculoskeletal tissues, including bone, cartilage, and tendon. Indeed, loss of PC1 (encoded by *Pkd1*) in mice leads to abnormal bone development, osteopenia, and impaired differentiation of osteoblasts.¹³⁴ The pathways activated by primary cilia in bone appear independent of Ca²⁺ flux¹³⁵ and are mediated by adenylyl cyclase 6 and cAMP.¹³⁶ In tendons, primary cilia may have a homeostatic role, although functional studies have not been carried out.^{137–139} Generally, primary cilia in tendons are oriented in the direction of tensile loading, and changes to cilia orientation or length can be detected in response to external loading. Collectively, these data suggest a mechanism whereby cells in different tissues can respond to mechanical stimuli through various downstream signaling pathways.

Discussion

While there has been considerable success in identifying key molecular regulators of musculoskeletal development, the role of mechanical forces is less well understood and remains an intriguing challenge for orthopedic researchers. The findings discussed in this

review indicate that cellular forces at the microscale, as well as tissue-generated forces and muscle loading at the macroscale, guide numerous aspects of tissue differentiation and maturation during development. One difficulty in better understanding these phenomena is the difficulty in directly linking microscale to macroscale events. Bridging these two scales will require the use of emerging technologies, experimental manipulations, and computational models that predict cellular processes on the basis of mechanical inputs. Progress in this field may also inform translation of regenerative therapies into orthopedic practices. One of the major challenges in orthopedic tissue engineering is the inability to generate mature tissues with functional properties that approach native tissues, even in the presence of soluble morphogens known to induce tissue-specific differentiation. This challenge may be overcome by the application of the appropriate mechanical cues and microenvironmental contexts to regulate cell biology.^{140, 141}

Acknowledgments

This work was supported by funding from the National Institutes of Health (R01AR069537) to A.H.H.

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Figure 1.

(A) Mechanical forces regulate skeletal development via muscle contraction (initiating at ~ E13) or via radial stresses induced by perichondral constraint (~ E11.5). (B) Mature enthesis architecture is composed of bone, mineralized fibrocartilage, fibrocartilage, and tendon that is optimized to minimize stress concentrations during load transfer. (C) Joint induction begins with specification of interzone cells at the presumptive joint site, which is distinguished by loss of chondrogenic markers and upregulation of interzone markers. Cavitation of the joint subsequently separates the skeletal elements.

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Figure 2.

Schematic of cell–ECM mechanotransduction. Cells respond via integrin signaling to high and low stresses based on underlying substrate stiffness. Stiff environments recruit integrin and FAK complexes that form the adhesome, which can potentially signal via several downstream pathways, notably RhoGTPase signaling. Cytoskeletal actin mediate force transduction from the cell membrane to the nucleus, leading to reorganization of nuclear architecture.

Figure 3. Schematic of cell–cell mechanotransduction. Binding of cadherins initializes cell–cell mechanotransduction, which recruits adhesion proteins that facilitate recruitment of cytoskeletal actin and other intracellular signaling molecules similar to cell–ECM transduction.

Figure 4.

Schematic of primary cilia-mediated mechanotransduction. Primary cilia are composed of a central singular axoneme that serves as scaffolding for the intraflagellar transport proteins that move up and down the axoneme. At the base of the cilia are basal body proteins that form BBsomes, which are required for formation and maintenance of the cilia. Mechanical deflection of primary cilia leads to activation of downstream signaling pathways.

Table 1

Pharmaceutical agents and genetic mutants for investigating mechanoregulation in chick and mouse, respectively.

Neuromuscular modulator	Action
Decamethonium	Rigid paralysis
Botulinum toxin	Flaccid paralysis
Succinylcholine	Flaccid paralysis
Tubocurarine	Flaccid paralysis
Mouse genotype	Mouse phenotype
<i>Pax3^{Sp/Sp} (Spotch)¹⁵</i>	Lack of limb muscle; lethal by E14.5
<i>Pax3^{Spd/Spd} (Spotch delayed)¹⁶</i>	Lack of limb muscle; lethal by E18
<i>MyoD^{-/-} ; Myf-5^{-/-19}</i>	Lack of organismal skeletal muscle
<i>MyoD^{-/-} ; Myf-5^{-/+19}</i>	Reduced organismal skeletal muscle mass
Muscular dysgenesis (<i>mdg/mdg</i>) ²¹	Muscle paralysis