Title

Changing While Staying the Same: Preservation of Structural Continuity During Limb Evolution by Developmental Integration.

Journal

Integrative and Comparative Biology, 57(6)

ISSN

1540-7063

Authors

Tsutsumi, Rio
Tran, Mai P
Cooper, Kimberly L

Publication Date

2017-12-01

DOI

10.1093/icb/icx092

Peer reviewed

Integrative and Comparative Biology

Integrative and Comparative Biology, volume 57, number 6, pp. 1269–1280
doi:10.1093/icb/icx092

Society for Integrative and Comparative Biology

SYMPOSIUM

Changing While Staying the Same: Preservation of Structural Continuity During Limb Evolution by Developmental Integration

Rio Tsutsumi, Mai P. Tran and Kimberly L. Cooper[1]

Division of Biological Sciences, University of California San Diego, La Jolla, CA 92093-0380, USA

From the symposium "Physical and Genetic Mechanisms for Evolutionary Novelty" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 4–8, 2017 at New Orleans, Louisiana.

[1]E-mail: kcooper@ucsd.edu

Synopsis More than 150 years since Charles Darwin published "On the Origin of Species", gradual evolution by natural selection is still not fully reconciled with the apparent sudden appearance of complex structures, such as the bat wing, with highly derived functions. This is in part because developmental genetics has not yet identified the number and types of mutations that accumulated to drive complex morphological evolution. Here, we consider the experimental manipulations in laboratory model systems that suggest tissue interdependence and mechanical responsiveness during limb development conceptually reduce the genetic complexity required to reshape the structure as a whole. It is an exciting time in the field of evolutionary developmental biology as emerging technical approaches in a variety of non-traditional laboratory species are on the verge of filling the gaps between theory and evidence to resolve this sesquicentennial debate.

Introduction

Imagine a human arm morphing into the wing of a bat. Each finger grows long and slender. Webbing extends to the tips. Blood vessels and nerves weave out into the spaces between. Long tendons extend to connect muscle to bone across joints that adjust their range of motion to support powered flight.

Opponents of Charles Darwin found the limb to be one of the greatest challenges to his theory that natural selection, acting gradually on small variations, could be sufficient to generate as complex a structure as a wing (Darwin 1859; Mivart 1871; Gould 1985). Functional morphologists have long tackled the problem of identifying the adaptive advantage of "incipient", or partial structures, but criticisms are also grounded in the assumption that morphological restructuring of limbs and other structures requires simultaneous and sometimes massive remodeling of multiple tissues in a coordinated manner (Mivart 1871). From this perspective, it seems almost implausible that limbs acquire qualitatively different morphologies or functions from their ancestral state, and yet the limb has been modified in myriad ways in all vertebrate lineages.

The idea that restructuring requires genetic changes independently affecting multiple tissues stems from early ideas that the embryo forms as an assemblage of parts, each manifesting its own intrinsic destiny. We now know that development proceeds by a combination of intrinsic (or autonomous) and extrinsic (or nonautonomous) mechanisms. Component parts are in constant communication, and abnormalities or deficiencies are often compensated by neighboring cells and tissues. Together, autonomous and non-autonomous mechanisms contribute to both the robustness and malleability of the developing organism (Lawrence and Levine 2006).

Kirschner and Gerhart (2005) argued that the interdependence of developing tissues confers plasticity of organ morphology, and that if this plasticity is evolutionarily conserved, functionally integrated structures are produced in response to the change in a component tissue. This process, which Kirschner and Gerhart termed "exploratory behavior," is not only important for preserving function in the face of environmental and genetic variation, but may also have a crucial role in producing novelty by amplifying the effect of change

Advance Access publication September 13, 2017

occurring in one tissue or cell type (Müller 1990; West-Eberhard 2003; Kirschner and Gerhart 2005).

Tissue interdependence in development and evolution has been perhaps best illustrated by embryonic manipulations to produce chimeric faces of the "quck" and "duail" (Schneider and Helms 2003). Interspecific transplants of neural crest cells from the quail to duck and vice versa showed there is an intrinsic program by which the neural crest cells form the beak or bill of their species of origin. These experiments also showed the ability of neural crest-derived structures to non-autonomously impart donor-species characteristics on host-derived tissues. For example, the gene expression of the overlying ectoderm and morphology of the egg tooth match the donor rather than the host. Together, these outcomes demonstrate both the autonomous and non-autonomous properties of embryo development and illustrate how genetic change in one tissue (the neural crest-derived skeleton) can influence the development of another (the ectoderm-derived egg tooth).

At this small scale, neighboring integrated tissues function as the "environment" that mutually influences morphological development. On a grander scale, features of the developing organism are so malleable that behavior can have a great impact on form and function. Mary West-Eberhard termed this effect "phenotypic accommodation" and described it as the "adaptive mutual adjustment among various parts during development without genetic change" (West-Eberhard 2003). She argued that organisms could adjust their physiology or morphology in a single generation in order to adapt to an environmental change. If mutations occur that "hardwire" the phenotypic outcome, these allele frequencies could increase in a population by natural selection in a process called "genetic assimilation" (Waddington 1953; West-Eberhard 2003, 2005).

A striking example of phenotypic accommodation occurred when a two-legged goat, born without forelegs, learned to hop upright. The Dutch anatomist, Everhard J. Slijper, acquired this remarkable animal and, after an unfortunate accident resulting in its death, described its unique anatomical features. Slijper's goat had developed a suite of characteristics that appear convergently in naturally bipedal species, including modifications to its spine, ribcage, hindlimbs, and musculature (Slijper 1942a, 1942b). This example highlights the potential evolutionary role of behavior in shaping the musculoskeletal system through the coordinated change of multiple structures and tissue types. If locomotor changes and their concomitant morphological effects are adaptive, and if they alter an animal's occupied niche, such changes may also influence and accelerate the trajectory of subsequent evolutionary events (Diogo 2017).

Although these examples support the concepts of exploratory behavior and phenotypic accommodation, the mechanisms acting to shape the limb during evolution remain largely unknown. Since developmental integration of multiple tissues in the limb likely underlies evolution of the same complex structures, we can make great strides by considering lessons from experimental embryology. Here, we detail the origins and developmental interdependence of the skeleton, muscle, and connective tissues of the vertebrate limb. While much is known of the molecular pathways intrinsic to the development of each tissue, we limit our discussion to communications between tissues and to the environmental effects of mechanical force and locomotion. We highlight opportunities where tissue integration and responsiveness to mechanical force may maintain structural and functional continuity as the limb is transformed by natural selection. The diverse outcomes of selection for novel limb structures and functions are innumerable. We therefore leave the discussions of specific morphologies to others, including the complementary article on the evolution of avian limbs by Vargas and colleagues that also appears in this issue (citation to be added in press).

Developmental origins of the limb and its composite tissues

The developing limbs emerge from the flank of the embryo as buds that, over subsequent days, elongate and widen at their tips to form the hands and feet. At bud initiation, lateral plate mesoderm cells in an epithelial sheet lose contacts with their neighbors and rearrange into a mesenchymal bulge ensheathed in a thin cup of ectoderm (Gros and Tabin 2014). The lateral plate-derived mesenchymal cells of the limb bud ultimately produce the skeleton, joints, tendons, ligaments, dermis, and muscle connective tissues while the ectoderm gives rise to hair, feathers, or scales. Limb muscle and vascular endothelial cells originate in the somitic mesoderm and migrate into the emerging limb bud (Chevallier et al. 1977; Christ et al. 1977; Lance-Jones 1988) (Fig. 1).

Formation of the limb skeleton initiates as a Y-shaped condensation of limb bud mesenchyme (the humerus and radius/ulna) that further branches distally into each of the digits (Fell 1925; Thorogood and Hinchliffe 1975). Since this cartilaginous anlage provides a template for the future limb skeleton, the pattern of pre-cartilage condensation in the nascent

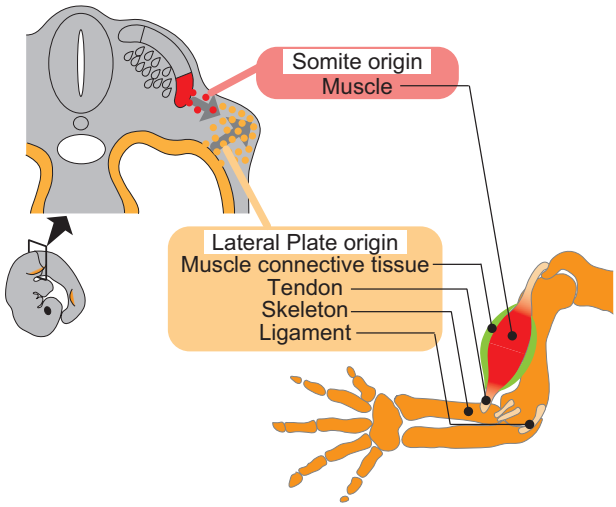

Fig. 1 Tissue origins of the musculoskeletal system. Lateral plate mesoderm cells in the presumptive limb region delaminate and give rise to limb bud mesenchymal cells. The lateral plate-derived mesenchymal cells will ultimately produce muscle connective tissues, tendons, skeleton, and ligaments in the limb. In contrast, limb muscles originate from precursor cells that delaminate from ventral–lateral somite and migrate into limb bud.

bud limits the potential for skeletal element number and organization. It has been proposed that cartilage pattern can self-organize as a result of reaction–diffusion interactions among mesenchymal cells without requiring prior heterogeneity of the cells (Zhu et al. 2010; Sheth et al. 2012; Raspopovic et al. 2014). Therefore the number and pattern of skeletal elements can be theoretically determined in a tissue-autonomous manner.

Joints are formed at the articulations between skeletal elements and are made up of force-absorbing articular chondrocytes (permanent cartilage) that secrete an oily lubricating substance (Schmidt et al. 2007). The nascent joint is formed from a region of flattened mesenchymal cells, called the joint interzone, that is delineated within the cartilage anlagen (Holder 1977; Mitrovic 1978). Lineage tracing experiments show that interzone cells give rise to the articular chondrocytes, synovial linings, and ligaments that connect skeletal elements across the joint (Koyama et al. 2008; Shwartz et al. 2016).

While the two major fibrous connective tissues, ligaments and tendons, have a very similar structural composition of sparse cells embedded in a dense network of extracellular matrix composed primarily of Type I Collagen, they have different origins in the limb bud. Ligaments are derived from joint interzone cells, while tendon progenitor cells are induced in the dorsal and ventral mesenchyme that lies between the ectoderm and condensing cartilage (Kardon 1998; Schweitzer et al. 2001; Watson et al. 2009). Within the tendon primordia, cells organize into longitudinal rows, and the orientation of these rows determines the direction of collagen fibers (McNeilly et al. 1996; Kardon 1998). Small tendon fibers organize into limb-specific patterns by embryonic day 12 in the mouse, and final pattern is formed by embryonic day 16.5 (Schweitzer et al. 2001; Watson et al. 2009).

Muscle connective tissue is a thin layer of cells embedded in extracellular matrix that ensheaths individual muscle cells and bundles of myofibers (Light and Champion 1984). Like the cartilage, joints, ligaments, and tendons, limb muscle connective tissue is derived from limb bud mesenchymal cells that originate in the lateral plate mesoderm (Chevallier et al. 1977). Muscle connective tissue progenitors cluster in the limb bud in a pre-pattern reflective of later muscle pattern and are first identifiable by expression of the transcription factor *Tcf4* (Kardon et al. 2003). This fibrous matrix, mainly comprising Type I Collagen, provides a structure and chemical environment for proper muscle function and transmits the collective forces of muscle fiber contraction (Kardon et al. 2003; Mathew et al. 2011).

Muscle, ensheathed in its connective tissue and connected to bones by tendons, pulls the levers of the limb skeleton to propel an animal in motion. However, unlike the other tissues in this integrated musculoskeletal unit that are derived from lateral plate mesoderm, muscle cells delaminate from the ventral–lateral somite and migrate into the nascent limb bud (Chevallier et al. 1977; Christ et al. 1977; Hayashi and Ozawa 1991). Once in the limb, the migrating population segregates into the ventral (future flexor) and dorsal (future extensor) muscle masses (Hayashi and Ozawa 1991). These muscle masses undergo further segregation to form the superficial and deep muscle layers, which then subdivide again into distinct muscles present in the adult (Hayashi and Ozawa 1991; Wortham 1948). The formation of individual muscles within this pattern is initiated when embryonic myoblasts fuse to form multinucleated myofibers in a process called "primary myogenesis". Mature muscle is built from nascent muscle by the later addition of fetal and neonatal myoblasts that fuse to primary myofibers or fuse to form new secondary myofibers (Abmayr and Pavlath 2012).

Developmental interdependence and integration

Muscle–muscle connective tissue

Although the degree and nature of dependency varies, the development of each composite tissue of

the integrated musculoskeletal system relies on the development of other tissues and on their mechanical function as a unit. Muscle seems to be the most reliant on extrinsic cues. While primary and secondary myogenesis occur autonomously, the spatial pattern of adult limb muscle anatomy is not specified by the myoblast population itself (Kardon 1998). In fact, since cervical and interlimb somites engrafted to the limb level give rise to normal limb muscle anatomy, it is clear that the pattern of adult muscle is largely a result of signals in the surrounding limb mesoderm (Chevallier et al. 1977; Christ et al. 1977). Indeed, quail myogenic progenitors that are placed in a chick wing bud devoid of muscle are organized to form muscle with characteristics of the chick host, including a muscle that is ordinarily absent in the quail (Grim 1991). It is therefore easy to see how muscle development could secondarily accommodate evolutionary changes in other limb tissues by the addition or loss of individual muscles or by changing their connections to the skeleton.

Tcf4, a transcription factor downstream of the *Wnt/β-catenin* signaling pathway, was identified in a quest for the extrinsic determinants of limb muscle pattern. Its muscle-like pattern of expression in muscle connective tissue progenitors persists even in limbs that develop without muscle (Kardon et al. 2003). Together, these data suggested that the muscle connective tissue, perhaps through activation of the *Wnt/β-catenin* pathway, provides the prepattern for developing muscle. Indeed, misexpression of activated *Wnt/β-catenin* in limb bud mesoderm is sufficient to induce ectopic muscle (Kardon et al. 2003), and *Tcf4* mutant mice have abnormal muscle truncations and segregations and altered connections to the skeleton (Mathew et al. 2011). Although the mechanisms that establish a *Wnt/β-catenin*-dependent pattern of muscle connective tissue remain unknown, evolutionary alterations to this pattern are expected to be transmitted from muscle connective tissue to repattern muscle anatomy.

However, muscle pattern is not a simple one-way instruction from the muscle connective tissue to muscle. Although several lines of evidence support the hypothesis that the blueprint for muscle resides in the limb bud mesoderm, vascular endothelial cells derived from the somite are also required for normal segregation of muscle masses into individual muscles (Tozer et al. 2007). Ectopic vasculature inhibits myoblast differentiation and promotes the aggregation of Type 1 Collagen-rich muscle connective tissue, and endothelial cells are necessary for normal muscle segregation. Tissue grafting experiments indicate that the stereotyped pattern of blood vessels throughout the body is largely dependent on the response of vascular cells to extrinsic cues in their environment (Noden 1989). However, the anatomical relationship between muscle and muscle connective tissue may be relayed and reinforced by the vasculature that surrounds and nourishes these tissues.

The interdependence of muscle and muscle connective tissue extends beyond development of early pattern to cell type differentiation and maintenance. In addition to a role in defining muscle fiber type (Mathew et al. 2011), muscle connective tissue fibroblasts are required for the maintenance of muscle satellite cells. Ablation of muscle connective tissue fibroblasts leads to premature differentiation of the resident muscle stem cells resulting in smaller regenerated myofibers (Murphy et al. 2011). In turn, muscle cells secrete the enzyme lysyl oxidase, which directly or indirectly represses *TGF*β signaling in muscle connective tissue to limit cell number and collagen deposition (Kutchuk et al. 2015). The prolonged interaction of muscle and muscle connective tissue during development thus ensures the appropriate balance of these two tissues for proper function of the integrated unit and for coordinated evolutionary modification.

Muscle–tendon

Muscle and tendon develop in close association in chick and mouse limbs (Kardon 1998; Watson et al. 2009), and muscle extends into ectopic regions in the absence of tendon (Kardon 1998). This suggests that tendon, in addition to muscle connective tissue and vasculature, establishes the spatial limits of muscle and perhaps influences muscle segregation. Tendon also relies reciprocally on limb muscle for proper pattern and maintenance (Christ et al. 1977; Kieny and Chevallier 1979; Kardon 1998; Schweitzer et al. 2001).

However, proximal and distal limb tendons respond differently to the lack of muscle; tendons located within the arm and leg fail to segregate and subsequently degenerate while those in the hand and foot continue to form but fail to mature (Kardon 1998). Similarly, proximal and distal tendons respond differently to the influence of condensing cartilage. While proximal tendons form normally in the absence of humerus, radius, and ulna, distal tendons of the hands and feet fail to form in the absence of digit cartilages (Huang et al. 2015). Ectopic digits in mouse (Huang et al. 2015) or chick (Hurle et al. 1990) promote the formation of associated ectopic tendons. Therefore cartilage appears to be both necessary and sufficient for tendon

formation in the hands and feet, which may influence the co-evolution of connective tissues together with their associated digits. The different requirements for cartilage in the distal versus proximal limb may be explained in part by the modular development of tendon whereby distal and proximal tendons form independently and later join to form functional tendon architecture (Huang et al. 2013, 2015).

Much like muscle connective tissue and muscle, tendon and muscle also appear to signal to one another to reciprocally promote growth. The bone morphogenetic protein (*Bmp*) pathway is active in muscle cells at the interface with tendon, and tendon expresses *Bmp4*. Experimentally increasing *Bmp* signaling in muscle activates progenitor proliferation and increases muscle mass while blocking the *Bmp* pathway decreases the number of muscle progenitors and differentiated myofibers (Wang et al. 2010). In the chick, apoptotic cell death eliminates myofibers that fail to make appropriate muscle–tendon connections, and this seems to be mediated, at least in part, by retinoic acid (Rodriguez-Guzman et al. 2007). These and other signaling pathways may together determine the relative size of muscle and tendon to ensure an appropriate functional connection.

During juvenile development, muscle and tendon growth continues to be accommodative such that the length of muscle contraction will optimally produce the full range of motion about the joint. Surgical release of the tibialis anterior tendon by clipping the crural ligament in young rabbits changes the effective tendon angle and increases the distance over which the muscle must contract to exact a full range of joint motion. Over the subsequent year, the muscle elongates and the tendon shortens to increase the length of muscle contraction to recover the full range of motion (Crawford 1954). Altogether, these interactions illustrate how non-autonomous accommodative changes can occur to maintain functional integrity of the muscle–tendon unit in an organism over its lifetime and in the evolutionary transformation of species morphology.

Mechanical force—skeletal growth

Once the number and positions of the skeletal elements are autonomously established, each skeletal element is subjected to numerous modifications from the shape of the pre-cartilage condensation to the final adult morphology. These modifications include ossification to establish optimal bone mineral density, longitudinal growth, circumferential growth and curvature, and enthesis, and joint formation. In addition to biochemical factors, mechanical stimuli influence each of these modifications in response to the loads associated with animal locomotion and behavior (Frost 2000; Pollard et al. 2014). Hence, since the largest loads come from muscle contractions via tendon attachments (Burr 1997), musculoskeletal integration directly connects animal behaviors to morphogenesis of the limb skeleton. Here, we focus on the extrinsic effect of muscle activity on the shape of the skeleton.

Enlarged hypertrophic chondrocytes near the junction of cartilage and bone secrete a mineralized matrix that forms the scaffold for mature bone. These large cells mature from small, flattened proliferative chondrocytes that are aligned into longitudinally oriented columns. Formation and maintenance of columns involves the intercalation of a series of clonally related chondrocytes and is essential for proper longitudinal skeletal growth. The absence or paralysis of limb muscle in mouse and chick results in shortened and misaligned proliferative chondrocyte columns in the growth plate and limbs that are often shortened, illustrating the dependence of skeletal elongation on proper muscle function (Hall and Herring 1990; Hosseini and Hogg 1991a; Germiller and Goldstein 1997; Osborne et al. 2002; Rot-Nikcevic et al. 2006; Gomez et al. 2007; Nowlan et al. 2010; Shwartz et al. 2012).

Bone modeling of the mineralized scaffold by osteoblasts starts at the mid-shaft of each of the cartilaginous anlagen and moves outward following a wave of cartilage maturation. In both mouse and chick, limb muscle contraction is also required for the normal initiation and expansion of ossification centers (Hosseini and Hogg 1991a; Nowlan et al. 2008a, 2008b, 2010). Bone is remodeled throughout adult life by osteoclasts that resorb bone and osteoblasts that deposit new mineralized matrix, and the relative activity of these two cell types determines bone mineral density. Optimal bone mineral density is a delicate balance between the amount of bone required to structurally support a body in motion countered by the cost to transport the weight of excessive bone. Several lines of evidence, including clinical correlations between muscle strength and bone mineral density in humans (Snow-Harter et al. 1990), suggest that biomechanical force exerts regional-specific influences on bone mineral density to accommodate stresses and strains associated with physical activity.

As each limb skeletal element elongates and ossifies, it also increases its circumference while maintaining the appropriate cortical bone thickness. Circumferential growth is controlled by periosteal

(outer membrane) bone deposition by osteoblasts and endosteal (inner membrane) bone resorption by osteoclasts. However, circumferential growth is not uniform. Anisotropic growth produces non-uniform cross-sectional shapes that are associated with compression and tension forces produced by mechanical load. Indeed, developing bone is so sensitive to mechanical force that anisotropic growth is initiated by fetal movement *in utero* (Sharir et al. 2011). Animals that are paralyzed during development have cylindrical limb bones suggesting muscle contraction is essential for anisotropic growth (Pai 1965; Hosseini and Hogg 1991a; Rodríguez et al. 1992; Gomez et al. 2007; Sharir et al. 2011).

Together with anisotropic circumferential growth, longitudinal curvature contributes to the strength of bone under physiological conditions (Sharir et al. 2011), and curvature of long bones is seen throughout terrestrial animals (Bertram and Biewener 1992). The development of curvature is also dependent on muscle contraction, since animals that develop without muscle or with paralyzed muscles develop straight skeletal elements (Lanyon 1980; Hall and Herring 1990; Rot-Nikcevic et al. 2006; Gomez et al. 2007). The functional advantage of curvature is paradoxical, since curvature compromise the loading capacity of the bone in a direction orthogonal to physiological load (Bertram and Biewener 1988; Sharir et al. 2011; Jade et al. 2014). However, curvature itself may increase the predictability of mechanical load in contrast to straight bones that may encounter more variable forces (Bertram and Biewener 1988, 1992). Additionally, the forces of muscle contraction acting on a curved element likely counter the bending strains induced by longitudinal curvature (Jade et al. 2014; Milne 2016).

Mechanical force—shaping of entheses

In addition to its influence on the longitudinal and circumferential shape of the bone, mechanical force also promotes the growth of bone at tendon attachment sites, the entheses, which protect the bone against the highest localized muscle contractile force. Formation and maintenance of the enthesis is highly sensitive to the interaction with tendons and muscles (Zelzer et al. 2014), and the enthesis progenitor cells are thought to be dedicated to the accommodative adaptation of bone in response to mechanical load (Blitz et al. 2013).

The mature entheses are broadly classified as fibrous or fibrocartilaginous (Benjamin et al. 2002). The fibrous entheses are periosteal attachments and typically insert into the metaphysis or diaphysis of long bones. The more common form, fibrocartilaginous entheses typically insert into the epiphysis or bone ridge at the end of a long bone. After the formation of a primordial tendon-to-bone attachment, the fibrocartilaginous tendon entheses are mineralized so that tendon-to-bone insertion is composed of a structural gradation of tissues with different biomechanical properties: tendon, fibrocartilage, mineralized fibrocartilage, and bone (Schwartz et al. 2012; Dyment et al. 2015). This graded structure is important for mechanical strength of the enthesis to prevent tear at the interface of two materials with different physical features, namely, stiff brittle bone and tough compliant tendon (Thomopoulos et al. 2010; Lu and Thomopoulos 2013).

Although the initiation of the enthesis and the perinatal development of the structure are entirely dependent on biochemical signals not discussed here (Blitz et al. 2009, 2013; Sugimoto et al. 2013), its maturation requires muscular contraction (Pai 1965; Hall and Herring 1990; Hosseini and Hogg 1991b; Rot-Nikcevic et al. 2006; Blitz et al. 2009; Nowlan et al. 2010). When mechanical loading from muscle activity is reduced, enthesis maturation is delayed with reduced mineral deposition and reduced fibrocartilage formation (Thomopoulos et al. 2007; Kim et al. 2010; Schwartz et al. 2013). The defect caused by unloading is likely due to high osteoclast activity, since osteoclast inhibition by alendronate partially rescues the demineralization phenotype (Tatara et al. 2014). Paralysis also leads to upregulation of *Hedgehog* signaling activity in the enthesis and an increase in the *Hedgehog* responsive cell population that gives rise to enthesis fibrocartilage cells. Together, this suggests the mechanical force is converted to a biochemical signal that decreases *Hedgehog* signaling and responsiveness. Decreased *Hedgehog* activity is important to allow fibrocartilage differentiation and to prevent remodeling by osteoclasts leading to structural reinforcement of a mineralized enthesis (Schwartz et al. 2015).

Mechanical force—joint morphogenesis

Together with the effects of muscle load on the length and shape of individual skeletal elements, muscle contraction is also required to maintain and shape the joint articulation and to form the sesamoids that are embedded in connective tissue surrounding the joints. (Hamburger and Waugh 1940; Lelkes 1958; Drachman and Sokoloff 1966; Ruano-Gil et al. 1978; Mitrovic 1982; Hosseini and Hogg 1991b; Mikic et al. 2000; Osborne et al. 2002; Kahn et al. 2009; Kim et al. 2010; Nowlan et al. 2010, 2014; Roddy et al. 2011). Movement, not just

contraction, is necessary to activate the *Wnt/β-catenin* signaling pathway, which is required for the maintenance of joint progenitor cells (Hartmann and Tabin 2001; Guo et al. 2004; Später et al. 2006a, 2006b; Kahn et al. 2009). Accordingly, rigid paralysis or hypermobility can also cause improper joint cavitation (Ruano-Gil et al. 1985; Osborne et al. 2002), and the joint is so sensitively tuned to mechanical force that only physiological magnitudes of force applied to *in vitro* cultured limbs will recapitulate normal joint morphology (Chandaria et al. 2016).

The dependence on movement for their formation likely allows joints to maintain their structural integrity by adjusting to changing musculoskeletal morphologies. For example, mutant mice lacking all *Hox11* paralogous genes, a subset of the *Hox* transcription factors required for the development of segmented structures, have hypoplastic zeugopod elements (radius/ulna and tibia/fibula). The elbow and knee joints of these *Hox11* mutant mice are remodeled and reorganized to make reciprocally interlocking structures between the unaffected humerus/femur and the hypoplastic zeugopod (Koyama et al. 2010). Interestingly, these mice gain an ulnar patella, which is characteristic of elbow joints in some reptiles, amphibians, and birds. Similarly, when the forelimb is amputated at the elbow joint in amphibians, the joint is reintegrated between the remaining and regenerated skeletal elements by plastically adjusting its morphology in the context of early differences in shape and size (Tsutsumi et al. 2016, 2015).

Even subtle changes to behavior or locomotion can have regionalized effects on the direction and/or magnitude of forces compared with the ancestral state. These altered forces can non-autonomously impart substantial changes to the length, cross-sectional shape, and curvature of a long bone element and can reshape the entheses and joint articulations. Indeed, the remarkable plasticity of bone in response to mechanical load suggests the great variation throughout tetrapods in the shape of limb joint articulations, the curvature of the tibia, and the prominence of the cnemial crest, for example, may be the products of extrinsic effects.

Theoretical impact of musculoskeletal integration and mechanical adaptability on evolvability

While these experiments illustrate the interdependence of limb tissue development, phenotypes that result from the wholesale loss or disruption of muscle or cartilage, for example, are perhaps not representative of the adaptive phenotypes that occur by natural selection. The observed interdependence, however, can extend to the hypothesis that adaptive phenotypes resulting from genetic change affecting one tissue will spread to impart non-genetic changes in the form and/or function of other tissues. By extrapolation, these non-autonomous effects observed after experimental manipulation decrease the theoretical genetic complexity required to substantially reshape the limb as an evolutionarily malleable structure.

Moreover, if genetic changes affecting the limb change locomotor performance, there will be an associated change in the direction and magnitude of mechanical forces on bones that will further reshape skeletal morphologies. The secondary effects on morphology of an acquired locomotor function are seen in the adjustments made throughout the skeleton to meet the mechanical demands in Slijper's goat (Slijper 1942a, 1942b). Thus, interdependence of the skeleton, muscle, and connective tissues not only ensures that essential locomotor function can be maintained in the face of fluctuations in normal development, but locomotor function itself also amplifies the effect of change throughout multiple tissues of the limb and potentially contributes to complex adaptive phenotypes (Fig. 2).

How can we test the hypothesis that tissue interdependence and locomotor performance contribute to major morphological transformations during limb evolution? A thorough understanding of the development of the derived phenotype is the first step, but each tissue in an intact animal develops in time and space alongside all of the others. To test the developmental autonomy or non-autonomy of individual tissues within a derived phenotype, we must be able to isolate genetic effects to specific tissues in order to assess the non-autonomous impacts on other tissues. This is possible through the production of inter-specific chimeras or by imparting evolutionarily relevant genetic changes in specific tissues.

Interspecies chimerism involves combining the tissues of two species to generate an individual with features of both. This powerful approach has been applied to the generation of quail–duck chimeras with a goal of understanding the autonomous and non-autonomous aspects of craniofacial development. Donor neural crest autonomously determines the species-specific morphology of jaw skeletal elements and secondarily reshapes the jaw muscles and attachment sites of host-derived tissues (Schneider and Helms 2003; Tokita and Schneider 2009). In turn, the changes in muscle pattern alter the exertion of mechanical forces on the jaw skeleton resulting in

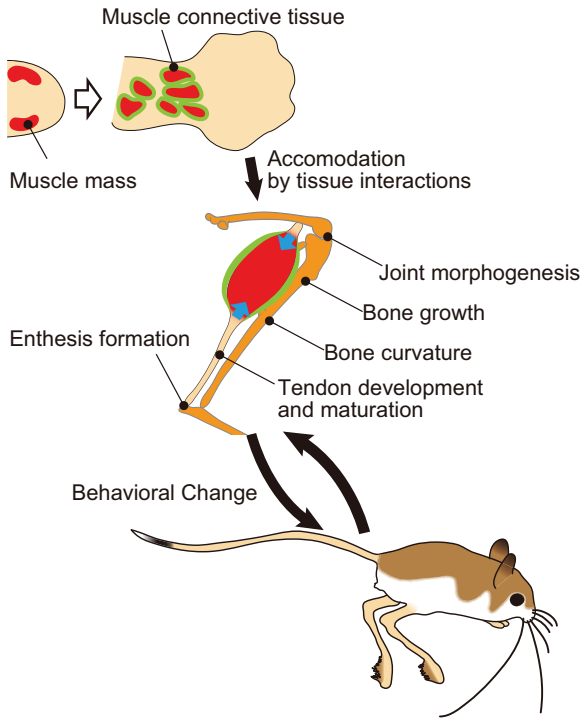

Fig. 2 Tissue interactions and accommodation in limb musculoskeltal development. Dorsal and ventral muscle masses in the limb bud are segregated and shaped into individual muscle groups by the lateral plate-derived connective tissues, especially muscle connective tissues. Once muscle, tendon, and skeleton are connected, mechanical forces from muscle contraction (arrows on muscle) stimulate bone shape modifications including joint morphogenesis, longitudinal and circumferential growth, curvature, and enthesis formation. If the evolutionary change in limb morphologies affects locomotor activity, the behavioral change will alter mechanical loading on bones and further reshape skeletal morphologies.

the loss of host-specific enthesis formation (Solem et al. 2011).

These experiments were accomplished by surgically transplanting embryonic tissues from one species into the orthotopic location of another. This surgical manipulation is possible in oviparous species, such as reptiles and birds, where the developing embryos are easily accessible and will continue to develop without maternal support. Similar transplants between quail and chick embryos contributed to the conclusion that signals in the lateral plate-derived limb mesoderm non-autonomously pattern species-specific differences in limb muscles (Grim 1991). Unfortunately, this approach is somewhat limited in its application to other tissues of the limb musculoskeletal system, since the cartilage, joints, tendon, and muscle connective tissue all derive from lateral plate mesoderm. It is not technically challenging to recombine somitic and lateral plate mesoderm prior to limb bud outgrowth, but once the progenitors of each tissue are intermixed in the limb, it is difficult to imagine isolating and transplanting only the nascent cartilages or connective tissues.

Recent advances in stem cell biology have opened opportunities to create interspecies chimeras between mammals that may circumvent these surgical obstacles. Using methods to induce pluripotency, combined with genetic ablation of a tissue niche in the mouse, it is possible to populate an entire organ or tissue type with cells of a different species (Kobayashi et al. 2010; Wu et al. 2017). While current applications are focused on the potential for human organ transplantation (Wu et al. 2017) and for conservation of endangered species (Honda et al. 2017), interspecies chimeras can potentially be used to test the autonomy and non-autonomy of tissue evolution. For example, populating the entire limb cartilage niche with pluripotent cells of bat origin may produce an elongated forelimb skeleton because of the evolution of autonomously functioning cartilage growth-promoting genes. The effects of these intrinsic cartilage programs on other tissues can then be assessed to determine how much of a wing is secondarily induced in the pattern of muscle, connective tissues, and the reciprocal influence on bone shape.

However, the bat and mouse diverged from a common ancestor approximately 95 million years ago and may be too distantly related for successful chimerism. To understand the evolution of development in a more closely related species, we have established a bipedal rodent, the lesser Egyptian jerboa (*Jaculus jaculus*) as a laboratory model (Cooper 2011). Jerboa hindlimbs have disproportionately elongated and fused metatarsals, a saddle-shaped femoral head, loss of lateral hindlimb digits, and anatomical replacement of foot muscles with tendon (suspensory ligaments). The jerboa shares a common ancestor with the laboratory mouse (*Mus musculus*) about 50–55 mya, and much of the extraordinary morphological divergence in the jerboa lineage has occurred within the last 35 million years (Fabre et al. 2012; Wu et al. 2012; Pisano et al. 2015). In contrast to the bat, for which little is known of the progression from terrestrial locomotion to powered flight, there are 33 species of extant jerboas with a spectrum of hindlimb morphotypes (Moore et al. 2015).

Using a genetic tissue ablation and stem cell-based interspecies repopulation approach in mouse, we can in theory replace the entire cartilaginous mouse limb skeleton with jerboa cells to determine whether digit loss by cell death occurs by cartilage-autonomous or non-autonomous mechanisms (Cooper et al. 2014)

and whether the rapid elongation of the limb skeleton secondarily drives elongation of the tendons and concomitant loss of intrinsic foot muscles. Repopulation of the *Tcf4*+ muscle connective tissue niche with cells derived from the jerboa would identify tissue-autonomous differences in the development of muscle connective tissue and the non-autonomous effect on the pattern of limb muscles.

As these methods are improved, similar approaches could be applied to a variety of species and morphologies to understand the complexity of limb development. Many features of the jerboa hindlimb appear convergently in phylogenetically distant cursorial animals, such as artiodactyls and perissodactyls (Clifford 2010). Frequent convergence may be a result of tissue interdependence accelerating the coordinated evolution of limb morphologies without requiring extensive genetic complexity. If true, then interspecies chimerism may reveal similar patterns of autonomous and non-autonomous development in distantly related taxa.

Ultimately, it will be important to identify how many mutations have accumulated to evolve a complex structure comprised of multiple tissues and how great are the effects of individual changes. Much of limb evolution has occurred over reproductively isolating distances, so quantitative trait analyses of hybrid genomes are not possible. Instead, approaches to engineer interspecies homologous replacements of the mouse genome will be important to test the sufficiency of specific genetic changes to impart derived limb phenotypes. These approaches have been successful in a few cases (Cretekos et al. 2008; Kvon et al. 2016). Improved genome engineering strategies using CRISPR/Cas9 will not only increase the speed and decrease cost of this approach but will also open avenues to performing sequence replacement in closely related non-model species.

Developmental integration of limb tissues and biomechanical feedback alone are not sufficient to explain the apparent sudden acquisition of complex morphologies, and it is likely that a complete fossil record would illustrate somewhat gradual metamorphoses from form to form. Indeed, character mapping of limb musculoskeletal traits in the jerboa clade suggests the transformation to obligate bipedalism involved multiple mutations affecting discrete (digit number) and continuous traits (bone fusion and skeletal elongation) (Moore et al. 2015). Developmental integration and mechanical responsiveness do, however, allow for the preservation of essential functions and provide a mechanism for "structural continuity" as the function of so called "incipient forms" shifts toward the derived state (e.g., powered flight or obligate bipedal locomotion) (Gould 1985). The challenge that lies ahead for the diverse field of evolutionary biology is to integrate our understanding of the morphological transformations and their adaptive advantage (comparative morphology, biomechanics, and ecology) with a deep understanding of the developmental genetic mechanisms that produce a striking array of complex phenotypes.

Funding

This work was supported by the Pew Biomedical Scholars Program, the Searle Scholars Program, and by a Packard Fellowship for Science and Engineering [to K.L.C.].

References

Abmayr SM, Pavlath GK. 2012. Myoblast fusion: lessons from flies and mice. Development 139:641–56.

Benjamin M, Kumai T, Milz S, Boszczyk BM, Boszczyk AA, Ralphs JR. 2002. The skeletal attachment of tendons—tendon "entheses." Comp Biochem Physiol A Mol Integr Physiol 133:931–45.

Bertram JEA, Biewener AA. 1992. Allometry and curvature in the long bones of quadrupedal mammals. J Zool 226:455–67.

Bertram JEA, Biewener AA. 1988. Bone curvature: sacrificing strength for load predictability? J Theor Biol 131:75–92.

Blitz E, Sharir A, Akiyama H, Zelzer E. 2013. Tendon-bone attachment unit is formed modularly by a distinct pool of *Scx*- and *Sox9*-positive progenitors. Development 140:2680–90.

Blitz E, Viukov S, Sharir A, Shwartz Y, Galloway JL, Pryce BA, Johnson RL, Tabin CJ, Schweitzer R, Zelzer E. 2009. Bone ridge patterning during musculoskeletal assembly is mediated through SCX regulation of Bmp4 at the tendon–skeleton junction. Dev Cell 17:861–73.

Burr DB. 1997. Muscle strength, bone mass, and age-related bone loss. J Bone Miner Res 12:1547–51.

Chandaria VV, McGinty J, Nowlan NC. 2016. Characterising the effects of in vitro mechanical stimulation on morphogenesis of developing limb explants. J Biomech 49:3635–42.

Chevallier A, Kieny M, Mauger A. 1977. Limb–somite relationship: origin of the limb musculature. J Embryol Exp Morphol 41:245–58.

Christ B, Jacob HJ, Jacob M. 1977. Experimental analysis of the origin of the wing musculature in avian embryos. Anat Embryol 150:171–86.

Clifford AB. 2010. The evolution of the unguligrade manus in artiodactyls. J Vertebr Paleontol 30:1827–39.

Cooper KL. 2011. The lesser Egyptian jerboa, *Jaculus jaculus*: a unique rodent model for evolution and development. Cold Spring Harb Protoc 2011:1451–6.

Cooper KL, Sears KE, Uygur A, Maier J, Baczkowski K-S, Brosnahan M, Antczak D, Skidmore JA, Tabin CJ. 2014. Patterning and post-patterning modes of evolutionary digit loss in mammals. Nature 511:41–5.

Crawford GNC. 1954. An experimental study of muscle growth in the rabbit. Bone Joint J 36-B:294–303.

Cretekos CJ, Wang Y, Green ED, Martin JF, Rasweiler JJ 4th, Behringer RR. 2008. Regulatory divergence modifies limb length between mammals. Genes Dev 22:141–51.

Darwin C. 1859. On the origin of species by means of natural selection, or, the preservation of favoured races in the struggle for life. London: John Murray.

Diogo R. 2017. Evolution driven by organismal behavior: a unifying view of life, function, form, mismatches and trends. 1st ed. New York (NY): Springer.

Drachman DB, Sokoloff L. 1966. The role of movement in embryonic joint development. Dev Biol 14:401–20.

Dyment NA, Breidenbach AP, Schwartz AG, Russell RP, Aschbacher-Smith L, Liu H, Hagiwara Y, Jiang R, Thomopoulos S, Butler DL, et al. 2015. *Gdf5* progenitors give rise to fibrocartilage cells that mineralize via hedgehog signaling to form the zonal enthesis. Dev Biol 405:96–107.

Fabre P-H, Hautier L, Dimitrov D, Douzery EJP. 2012. A glimpse on the pattern of rodent diversification: a phylogenetic approach. BMC Evol Biol 12:88.

Fell HB. 1925. The histogenesis of cartilage and bone in the long bones of the embryonic fowl. J Morphol 40:417–59.

Frost HM. 2000. The Utah paradigm of skeletal physiology: an overview of its insights for bone, cartilage and collagenous tissue organs. J Bone Miner Metab 18:305–16.

Germiller JA, Goldstein SA. 1997. Structure and function of embryonic growth plate in the absence of functioning skeletal muscle. J Orthop Res 15:362–70.

Gomez C, David V, Peet NM, Vico L, Chenu C, Malaval L, Skerry TM. 2007. Absence of mechanical loading in utero influences bone mass and architecture but not innervation in *Myod–Myf5*-deficient mice. J Anat 210:259–71.

Gould SJ. 1985. Not necessarily a wing. Nat Hist 94:12–9.

Grim M. 1991. Control of muscle morphogenesis and endplate pattern in limb muscle of avian chimeras. In: Developmental patterning of the vertebrate limb. A: Life sciences. New York (NY): Plenum Press. p. 293–7.

Gros J, Tabin CJ. 2014. Vertebrate limb bud formation is initiated by localized epithelial-to-mesenchymal transition. Science 343:1253–6.

Guo X, Day TF, Jiang X, Garrett-Beal L, Topol L, Yang Y. 2004. *Wnt/β-catenin* signaling is sufficient and necessary for synovial joint formation. Genes Dev 18:2404–17.

Hall BK, Herring SW. 1990. Paralysis and growth of the musculoskeletal system in the embryonic chick. J Morphol 206:45–56.

Hamburger V, Waugh M. 1940. The primary development of the skeleton in nerveless and poorly innervated limb transplants of chick embryos. Physiol Zool 13:367–82.

Hartmann C, Tabin CJ. 2001. *Wnt-14* plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell 104:341–51.

Hayashi K, Ozawa E. 1991. Vital labelling of somite-derived myogenic cells in the chicken limb bud. Roux's Arch Dev Biol 200:188–92.

Holder N. 1977. An experimental investigation into the early development of the chick elbow joint. Development 39:115–27.

Honda A, Choijookhuu N, Izu H, Kawano Y, Inokuchi M, Honsho K, Lee A-R, Nabekura H, Ohta H, Tsukiyama T, et al. 2017. Flexible adaptation of male germ cells from female iPSCs of endangered *Tokudaia osimensis*. Sci Adv 3:e1602179.

Hosseini A, Hogg DA. 1991a. The effects of paralysis on skeletal development in the chick embryo. II. Effects on histogenesis of the tibia. J Anat 177:169–78.

Hosseini A, Hogg DA. 1991b. The effects of paralysis on skeletal development in the chick embryo. I. General effects. J Anat 177:159–68.

Huang AH, Riordan TJ, Wang L, Eyal S, Zelzer E, Brigande JV, Schweitzer R. 2013. Repositioning forelimb superficialis muscles: tendon attachment and muscle activity enable active relocation of functional myofibers. Dev Cell 26:544–51.

Huang AH, Riordan TJ, Pryce B, Weibel JL, Watson SS, Long F, Lefebvre V, Harfe BD, Stadler HS, Akiyama H, et al. 2015. Musculoskeletal integration at the wrist underlies the modular development of limb tendons. Development 142:2431–41.

Hurle JM, Ros MA, Gañan Y, Macias D, Critchlow M, Hinchliffe JR. 1990. Experimental analysis of the role of ECM in the patterning of the distal tendons of the developing limb bud. Cell Differ Dev 30:97–108.

Jade S, Tamvada KH, Strait DS, Grosse IR. 2014. Finite element analysis of a femur to deconstruct the paradox of bone curvature. J Theor Biol 341:53–63.

Kahn J, Shwartz Y, Blitz E, Krief S, Sharir A, Breitel DA, Rattenbach R, Relaix F, Maire P, Rountree RB, et al. 2009. Muscle contraction is necessary to maintain joint progenitor cell fate. Dev Cell 16:734–43.

Kardon G. 1998. Muscle and tendon morphogenesis in the avian hind limb. Development 125:4019–32.

Kardon G, Harfe BD, Tabin CJ. 2003. A *Tcf4*-positive mesodermal population provides a prepattern for vertebrate limb muscle patterning. Dev Cell 5:937–44.

Kieny M, Chevallier A. 1979. Autonomy of tendon development in the embryonic chick wing. J Embryol Exp Morphol 49:153–65.

Kim HM, Galatz LM, Das R, Patel N, Thomopoulos S. 2010. Musculoskeletal deformities secondary to neurotomy of the superior trunk of the brachial plexus in neonatal mice. J Orthop Res 28:1391–8 (doi:10.1002/jor.21128).

Kirschner MW, Gerhart JC. 2005. The plausibility of life: resolving Darwin's dilemma. 1st ed. New Haven (CT): Yale University Press.

Kobayashi T, Yamaguchi T, Hamanaka S, Kato-Itoh M, Yamazaki Y, Ibata M, Sato H, Lee Y-S, Usui J, Knisely AS, et al. 2010. Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells. Cell 142:787–99.

Koyama E, Shibukawa Y, Nagayama M, Sugito H, Young B, Yuasa T, Okabe T, Ochiai T, Kamiya N, Rountree RB, et al. 2008. A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. Dev Biol 316:62–73.

Koyama E, Yasuda T, Minugh-Purvis N, Kinumatsu T, Yallowitz AR, Wellik DM, Pacifici M. 2010. *Hox11* genes establish synovial joint organization and phylogenetic characteristics in developing mouse zeugopod skeletal elements. Development 137:3795–800.

Kutchuk L, Laitala A, Soueid-Bomgarten S, Shentzer P, Rosendahl A-H, Eilot S, Grossman M, Sagi I, Sormunen R, Myllyharju J, et al. 2015. Muscle composition is

regulated by a Lox–*TGFβ* feedback loop. Development 142:983–93.
Kvon EZ, Kamneva OK, Melo US, Barozzi I, Osterwalder M, Mannion BJ, Tissières V, Pickle CS, Plajzer-Frick I, Lee EA, et al. 2016. Progressive loss of function in a limb enhancer during snake evolution. Cell 167:633–42. e11.
Lance-Jones C. 1988. The somitic level of origin of embryonic chick hindlimb muscles. Dev Biol 126:394–407.
Lanyon LE. 1980. The influence of function on the development of bone curvature. An experimental study on the rat tibia. J Zool 192:457–66.
Lawrence PA, Levine M. 2006. Mosaic and regulative development: two faces of one coin. Curr Biol 16:R236–9.
Lelkes G. 1958. Experiments in vitro on the role of movement in the development of joints. Development 6:183–6.
Light N, Champion AE. 1984. Characterization of muscle epimysium, perimysium and endomysium collagens. Biochem J 219:1017–26.
Lu HH, Thomopoulos S. 2013. Functional attachment of soft tissues to bone: development, healing, and tissue engineering. Annu Rev Biomed Eng 15:201–26.
Mathew SJ, Hansen JM, Merrell AJ, Murphy MM, Lawson JA, Hutcheson DA, Hansen MS, Angus-Hill M, Kardon G. 2011. Connective tissue fibroblasts and Tcf4 regulate myogenesis. Development 138:371–84.
McNeilly CM, Banes AJ, Benjamin M, Ralphs JR. 1996. Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions. J Anat 189(Pt 3):593–600.
Mikic B, Wong M, Chiquet M, Hunziker EB. 2000. Mechanical modulation of tenascin-C and collagen-XII expression during avian synovial joint formation. J Orthop Res 18:406–15.
Milne N. 2016. Curved bones: an adaptation to habitual loading. J Theor Biol 407:18–24.
Mitrovic D. 1982. Development of the articular cavity in paralyzed chick embryos and in chick embryo limb buds cultured on chorioallantoic membranes. Cells Tissues Organs 113:313–24.
Mitrovic D. 1978. Development of the diarthrodial joints in the rat embryo. Am J Anat 151:475–85.
Mivart SG. 1871. On the genesis of species. New York: Appleton.
Moore TY, Organ CL, Edwards SV, Biewener AA, Tabin CJ, Jenkins FA Jr, Cooper KL. 2015. Multiple phylogenetically distinct events shaped the evolution of limb skeletal morphologies associated with bipedalism in the jerboas. Curr Biol 25:2785–94.
Müller GB. 1990. Developmental mechanisms at the origin of morphological novelty: a side-effect hypothesis. In: Nitecki MH, editor. Evolutionary innovations. Chicago and London: University of Chicago Press. p. 99–130.
Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G. 2011. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development 138:3625–37.
Noden DM. 1989. Embryonic origins and assembly of blood vessels. Am Rev Respir Dis 140:1097–103.
Nowlan NC, Bourdon C, Dumas G, Tajbakhsh S, Prendergast PJ, Murphy P. 2010. Developing bones are differentially affected by compromised skeletal muscle formation. Bone 46:1275–85.
Nowlan NC, Chandaria V, Sharpe J. 2014. Immobilized chicks as a model system for early-onset developmental dysplasia of the hip. J Orthop Res 32:777–85.
Nowlan NC, Murphy P, Prendergast PJ. 2008a. A dynamic pattern of mechanical stimulation promotes ossification in avian embryonic long bones. J Biomech 41:249–58.
Nowlan NC, Prendergast PJ, Murphy P. 2008b. Identification of mechanosensitive genes during embryonic bone formation. PLOS Comput Biol 4:e1000250.
Osborne AC, Lamb KJ, Lewthwaite JC, Dowthwaite GP, Pitsillides AA. 2002. Short-term rigid and flaccid paralyses diminish growth of embryonic chick limbs and abrogate joint cavity formation but differentially preserve precavitated joints. J Musculoskelet Neuronal Interact 2:448–56.
Pai AC. 1965. Developmental genetics of a lethal mutation, muscular dysgenesis (*mdg*), in the mouse. Dev Biol 11:82–92.
Pisano J, Condamine FL, Lebedev V, Bannikova A, Quere J-P, Shenbrot GI, Pages M, Michaux JR. 2015. Out of Himalaya: the impact of past Asian environmental changes on the evolutionary and biogeographical history of Dipodoidea (Rodentia). J Biogeogr 42:856–70.
Pollard AS, McGonnell IM, Pitsillides AA. 2014. Mechanoadaptation of developing limbs: shaking a leg. J Anat 224:615–23.
Raspopovic J, Marcon L, Russo L, Sharpe J. 2014. Digit patterning is controlled by a *Bmp-Sox9-Wnt* Turing network modulated by morphogen gradients. Science 345:566–70.
Roddy KA, Prendergast PJ, Murphy P. 2011. Mechanical influences on morphogenesis of the knee joint revealed through morphological, molecular and computational analysis of immobilised embryos. PLoS One 6:e17526.
Rodriguez-Guzman M, Montero JA, Santesteban E, Gañan Y, Macias D, Hurle JM. 2007. Tendon–muscle crosstalk controls muscle bellies morphogenesis, which is mediated by cell death and retinoic acid signaling. Dev Biol 302:267–80.
Rodríguez JI, Palacios J, Ruiz A, Sanchez M, Alvarez I, Demiguel E. 1992. Morphological changes in long bone development in fetal akinesia deformation sequence: an experimental study in curarized rat fetuses. Teratology 45:213–21.
Rot-Nikcevic I, Reddy T, Downing KJ, Belliveau AC, Hallgrímsson B, Hall BK, Kablar B. 2006. *Myf5*−/−:MyoD−/− amyogenic fetuses reveal the importance of early contraction and static loading by striated muscle in mouse skeletogenesis. Dev Genes Evol 216:1–9.
Ruano-Gil D, Nardi-Vilardaga J, Teixidor-Johé A. 1985. Embryonal hypermobility and articular development. Acta Anat 123:90–2.
Ruano-Gil D, Nardi-Vilardaga J, Tejedo-Mateu A. 1978. Influence of extrinsic factors on the development of the articular system. Cells Tissues Organs 101:36–44.
Schmidt TA, Gastelum NS, Nguyen QT, Schumacher BL, Sah RL. 2007. Boundary lubrication of articular cartilage: role of synovial fluid constituents. Arthritis Rheum 56:882–91.
Schneider RA, Helms JA. 2003. The cellular and molecular origins of beak morphology. Science 299:565–8.
Schwartz AG, Lipner JH, Pasteris JD, Genin GM, Thomopoulos S. 2013. Muscle loading is necessary for the formation of a functional tendon enthesis. Bone 55:44–51.

Schwartz AG, Long F, Thomopoulos S. 2015. Enthesis fibrocartilage cells originate from a population of Hedgehog-responsive cells modulated by the loading environment. Development 142:196–206.

Schwartz AG, Pasteris JD, Genin GM, Daulton TL, Thomopoulos S. 2012. Mineral distributions at the developing tendon enthesis. PLoS One 7:e48630.

Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, Lassar A, Tabin CJ. 2001. Analysis of the tendon cell fate using *Scleraxis*, a specific marker for tendons and ligaments. Development 128:3855–66.

Sharir A, Stern T, Rot C, Shahar R, Zelzer E. 2011. Muscle force regulates bone shaping for optimal load-bearing capacity during embryogenesis. Development 138:3247–59.

Sheth R, Marcon L, Bastida MF, Junco M, Quintana L, Dahn R, Kmita M, Sharpe J, Ros MA. 2012. Hox genes regulate digit patterning by controlling the wavelength of a Turing-type mechanism. Science 338:1476–80.

Shwartz Y, Farkas Z, Stern T, Aszódi A, Zelzer E. 2012. Muscle contraction controls skeletal morphogenesis through regulation of chondrocyte convergent extension. Dev Biol 370:154–63.

Shwartz Y, Viukov S, Krief S, Zelzer E. 2016. Joint development involves a continuous influx of *Gdf5*-positive cells. Cell Rep 15:2577–87.

Slijper EJ. 1942a. Biologic-anatomical investigations on the bipedal gait and upright posture in mammals, with special reference to a little goat, born without forelegs. I. Proc K Ned Akad Wet 45:288–95.

Slijper EJ. 1942b. Biologic-anatomical investigations on the bipedal gait and upright posture in mammals, with special reference to a little goat, born without forelegs. II. Proc K Ned Akad Wet 45:407–15.

Snow-Harter C, Bouxsein M, Lewis B, Charette S, Weinstein P, Marcus R. 1990. Muscle strength as a predictor of bone mineral density in young women. J Bone Miner Res 5:589–95.

Solem RC, Eames BF, Tokita M, Schneider RA. 2011. Mesenchymal and mechanical mechanisms of secondary cartilage induction. Dev Biol 356:28–39.

Später D, Hill TP, Gruber M, Hartmann C. 2006a. Role of canonical *Wnt*-signalling in joint formation. Eur Cell Mater 12:71–80.

Später D, Hill TP, O'Sullivan RJ, Gruber M, Conner DA, Hartmann C. 2006b. *Wnt9a* signaling is required for joint integrity and regulation of *Ihh* during chondrogenesis. Development 133:3039–49.

Sugimoto Y, Takimoto A, Akiyama H, Kist R, Scherer G, Nakamura T, Hiraki Y, Shukunami C. 2013. *Scx*+/*Sox9*+ progenitors contribute to the establishment of the junction between cartilage and tendon/ligament. Development 140:2280–8.

Tatara AM, Lipner JH, Das R, Kim HM, Patel N, Ntouvali E, Silva MJ, Thomopoulos S. 2014. The role of muscle loading on bone (re)modeling at the developing enthesis. PLoS One 9:e97375.

Thomopoulos S, Genin GM, Galatz LM. 2010. The development and morphogenesis of the tendon-to-bone insertion—what development can teach us about healing. J Musculoskelet Neuronal Interact 10:35–45.

Thomopoulos S, Kim H-M, Rothermich SY, Biederstadt C, Das R, Galatz LM. 2007. Decreased muscle loading delays maturation of the tendon enthesis during postnatal development. J Orthop Res 25:1154–63.

Thorogood PV, Hinchliffe JR. 1975. An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. Development 33:581–606.

Tokita M, Schneider RA. 2009. Developmental origins of species-specific muscle pattern. Dev Biol 331:311–25.

Tozer S, Bonnin M-A, Relaix F, Di Savino S, García-Villalba P, Coumailleau P, Duprez D. 2007. Involvement of vessels and PDGFB in muscle splitting during chick limb development. Development 134:2579–91.

Tsutsumi R, Inoue T, Yamada S, Agata K. 2015. Reintegration of the regenerated and the remaining tissues during joint regeneration in the newt *Cynops pyrrhogaster*. Regeneration 2:26–36.

Tsutsumi R, Yamada S, Agata K. 2016. Functional joint regeneration is achieved using reintegration mechanism in *Xenopus laevis*. Regeneration 3:26–38.

Waddington CH. 1953. Genetic assimilation of an acquired character. Evolution 7:118–26.

Wang H, Noulet F, Edom-Vovard F, Le Grand F, Duprez D, Duprez D. 2010. *Bmp* signaling at the tips of skeletal muscles regulates the number of fetal muscle progenitors and satellite cells during development. Dev Cell 18:643–54.

Watson SS, Riordan TJ, Pryce BA, Schweitzer R. 2009. Tendons and muscles of the mouse forelimb during embryonic development. Dev Dyn 238:693–700.

West-Eberhard MJ. 2005. Phenotypic accommodation: adaptive innovation due to developmental plasticity. J Exp Zool 304B:610–8.

West-Eberhard MJ. 2003. Developmental plasticity and evolution. Oxford: Oxford University Press.

Wortham RA. 1948. The development of the muscles and tendons in the lower leg and foot of chick embryos. J Morphol 83:105–48.

Wu J, Platero-Luengo A, Sakurai M, Sugawara A, Gil MA, Yamauchi T, Suzuki K, Bogliotti YS, Cuello C, Morales Valencia M, et al. 2017. Interspecies chimerism with mammalian pluripotent stem cells. Cell 168:473–86.e15.

Wu S, Wu W, Zhang F, Ye J, Ni X, Sun J, Edwards SV, Meng J, Organ CL. 2012. Molecular and paleontological evidence for a post-cretaceous origin of rodents. PLoS One 7:e46445.

Zelzer E, Blitz E, Killian ML, Thomopoulos S. 2014. Tendon-to-bone attachment: from development to maturity. Birth Defects Res C Embryo Today 102:101–12.

Zhu J, Zhang Y-T, Alber MS, Newman SA. 2010. Bare bones pattern formation: a core regulatory network in varying geometries reproduces major features of vertebrate limb development and evolution. PLoS ONE 5:e10892.