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INSTRUCTIONAL REVIEW: RESEARCH

The importance of foetal movement for co-ordinated cartilage and bone development *in utero*

CLINICAL CONSEQUENCES AND POTENTIAL FOR THERAPY

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Construction of a functional skeleton is accomplished through co-ordination of the developmental processes of chondrogenesis, osteogenesis, and synovial joint formation. Infants whose movement *in utero* is reduced or restricted and who subsequently suffer from joint dysplasia (including joint contractures) and thin hypo-mineralised bones, demonstrate that embryonic movement is crucial for appropriate skeletogenesis. This has been confirmed in mouse, chick, and zebrafish animal models, where reduced or eliminated movement consistently yields similar malformations and which provide the possibility of experimentation to uncover the precise disturbances and the mechanisms by which movement impacts molecular regulation. Molecular genetic studies have shown the important roles played by cell communication signalling pathways, namely Wnt, Hedgehog, and transforming growth factor-beta/bone morphogenetic protein. These pathways regulate cell behaviours such as proliferation and differentiation to control maturation of the skeletal elements, and are affected when movement is altered. Cell contacts to the extracellular matrix as well as the cytoskeleton offer a means of mechanotransduction which could integrate mechanical cues with genetic regulation. Indeed, expression of cytoskeletal genes has been shown to be affected by immobilisation. In addition to furthering our understanding of a fundamental aspect of cell control and differentiation during development, research in this area is applicable to the engineering of stable skeletal tissues from stem cells, which relies on an understanding of developmental mechanisms including genetic and physical criteria. A deeper understanding of how movement affects skeletogenesis therefore has broader implications for regenerative therapeutics for injury or disease, as well as for optimisation of physical therapy regimes for individuals affected by skeletal abnormalities.

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Article focus

- Human skeletal malformations can result from reduced or absent mechanical stimulation *in utero*.

- Embryonic skeletal development involves temporal and spatial coordination of cartilage and bone tissue, which relies on networks of complex biochemical interactions within and between cells. Gene expression and the signalling environment are altered when mechanical stimulation is reduced.

- Research in this area has important implications for the development of new approaches, both preventive and therapeutic, to skeletal disorders.

Key messages

- Animal models and cell culture have provided evidence for the importance of appropriate mechanical stimulation during normal skeletal development.

- There is potential to harness this knowledge for regenerative therapies, recreating the required mechanical and signalling environment to guide the formation of robust cartilage and bone tissues from stem cells in culture.

- Foetal and newborn skeletal health depends on mechanical stimulation *in utero*, suggesting that physical therapy could correct or lessen the effects of infant skeletal malformations resulting from a range of movement-inhibiting conditions.

Introduction

The vertebrate skeleton is remarkably well constructed for support, protection, and movement. While the adaptability of the

mature skeleton in response to physical stimuli has been long accepted (e.g. loss of bone mass by astronauts; increased strength of bones in the dominant limbs of athletes), the role of movement in shaping the skeleton during embryogenesis has been acknowledged only relatively recently. A number of intriguing studies have emerged which have documented the clinical significance of mechanical stimulation to normal foetal skeletal development and the consequential impact of reduced foetal movement. Importantly, they have highlighted the role of functional contractile muscle in the transduction of dynamic physical loads to form bones and joints. Developing limbs experience a relatively large range of movement with respect to other skeletal elements. As such, they are particularly subject to altered mechano-stimulatory cues and make for a striking example of the importance of movement for proper embryonic patterning.

Mechanical stimuli have historically been recognised as vital to bone repair and maintenance, dating back to Julius Wolff's 1888 law which characterised bone strength as proportional to the physical loads placed upon the structure.[1,2] An understanding of bone as an adaptable, responsive material was expanded upon through Frost's mechanostat theory, wherein forces exerted on the skeleton by associated muscle direct a biochemical response which carries out the bone remodelling.[3] This idea refocused attention on the possibility that physical cues could directly influence molecular and cellular processes within tissues. However, it was not until recently that these concepts were considered in terms of skeletal development *in utero*. A wealth of new evidence currently exists to support mechanical stimulation as a vital feature of healthy embryonic development.

Construction of a sturdy yet dynamic skeletal system involves the interrelated processes of bone development and joint formation. In the appendicular skeleton, failure of synchronisation between the development of diarthroses (full-movement joints) and long bones results in an embryo with limited movement. The embryo's capacity to perform muscular movements will effectively feed back into further development and later remodelling of the skeleton. Thus, proper skeletal formation is enabled by, and in turn enables a full range of embryonic movement. Although the impact that movement has on skeletal development *in utero* has now been well demonstrated, our understanding of how mechanical stimuli influence genetic and molecular regulation of developmental processes is very limited. It is therefore important to determine the mechanisms of mechanotransduction involved in skeletal development, as they have a lifelong reach on form and function.

Understanding how mechanical stimuli generated by foetal movement impact skeletal development will augment our fundamental knowledge of tissue formation and has at least two important potential avenues for clinical application. First, in informing possible therapeutic interventions *in utero* where foetal movement is restricted and second, in improving strategies for bone and cartilage tissue regeneration from stem cells for skeletal regenerative therapies.

In this review, we synthesise current knowledge of skeletal development and the impact of mechanical stimulation in order to highlight current research aims as well as potential clinical and regenerative applications.

Foetal movement and clinical consequences of reduced movement

Muscle-controlled movement begins early and continues throughout embryonic development. In humans, the first foetal movement is recorded at nine weeks postmenstrual age (approximately Carnegie stage 18[4]) just after innervation of the forelimbs during Carnegie stages 14 to 17[5] as the skeletal rudiments are forming. The temporal relationship between movement and skeletal development hints at a close functional relationship.

In the 1970s, newborns with joint contractures, pulmonary hypoplasia, facial deformities and overall growth retardation were suggested by some to suffer from specific autosomal-recessive mutations, whereas others argued that this phenotype resulted from related, though discrete, disorders.[6-8] The discovery that application of a muscle relaxant to pregnant female rats could generate the same set of neonatal abnormalities led Moessinger[9] to formalise a description of a foetal akinesia deformation sequence (FADS) where inhibition of foetal movement for prolonged periods could result in developmental deformations. The underlying causes of FADS are diverse, comprising disorders of the central nervous system, musculature, and connective tissue, as well as extrinsic factors such as maternal drug use or illness or foetal crowding.[10] However, the common factor is reduced foetal movement leading to a similar spectrum of defects in bone and joint formation including hypomineralised and brittle bones prone to fracture, and contracture or dysplasia of the joints.[11-14] Reduced movement can lead to a condition called temporary brittle bone disease (TBBD)[15-17] which, as its name suggests, is a transient condition where bone fails to form properly during embryonic development and which affects newborn babies and young children, leaving them susceptible to non-accidental injury during their first year of life.[18] It is distinct from osteogenesis imperfecta (OI), also called brittle bone disease (BBD), where mutated collagen proteins build malformed bones, and which is a permanent condition.[19] TBBD generally regresses within the first year as bones are sufficiently strengthened by normal mechanical stimuli. Nonetheless, the transient state of heightened risk of fracture is dangerous and stressful for affected newborn babies and can lead to the added difficulty of suspected abuse from the parent or caregiver. Both the role of reduced foetal movement in causing TBBD and the subsequent recovery following post-natal movement

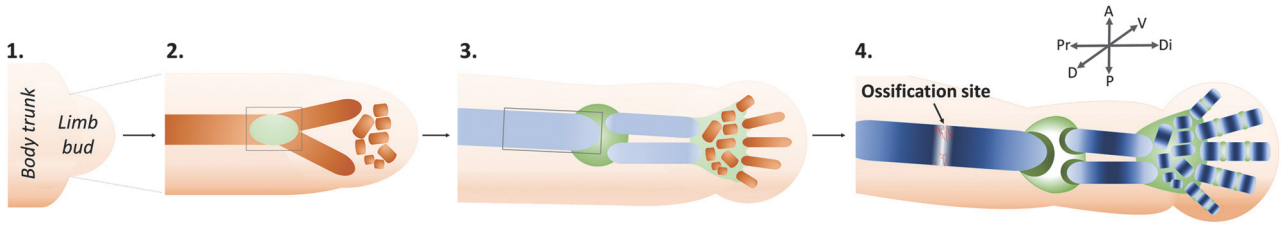

Fig. 1

Diagrams showing an overview of limb bud outgrowth and development. A generalised forelimb bud is represented, progressing through the major stages of skeletal development. Limbs first appear as buds of mesenchymal cells covered by ectoderm (1). Condensation of mesenchymal cells at the core of the bud is the earliest sign of the future skeletal elements (brown) e.g., humerus, radius, and ulna (2). Distal condensations, such as those of the carpals and digits, are progressively added. Future joint sites (green) become apparent within the mesenchymal condensations. The condensations differentiate into cartilage (light blue) (3). This cartilage matures, beginning at the mid-diaphysis of each rudiment (dark blue), proximal rudiments ahead of distal, (4) and is replaced by endochondral bone at the ossification site. Concurrently, joint interzones differentiate (3) and cavitate (4). Grey boxes in (2) and (3) indicate regions of synovial joint formation and endochondral ossification, respectively; these processes are described in detail in Figures 3 and 2, respectively.

indicate potential therapeutic interventions for affected infants.

Animal models of altered embryonic movement

The hypomineralised bones and unstable joints of infants born following reduced activity *in utero* indicate disturbances in skeletal development, and animal models provide a convenient means of investigating the precise steps of development altered in such an environment. Given the evolutionary conservation of musculoskeletal development among vertebrates, the two most established models of mammalian and avian development, namely mouse and chick, are well suited, with some limitations. Regular episodes of stereotypical muscular movement start at embryonic day (E) 12.5/Theiler stage (TS)[20] 20 in a mouse model and E 3.5/Hamburger and Hamilton (HH)[21] stage 21 in a chick model, which is concurrent with the appearance of limb skeletal rudiments and innervation in both species.[22-24] Muscle masses and tendons are gradually forming at these stages, so although it is not certain exactly when effective mechanical loads begin to be transmitted to cells of the cartilage anlagen,[25] movement occurs from early stages of appendicular skeletal development and is an integral feature of the system.

The mechanical environment of these model systems can be manipulated genetically and physically to examine embryonic skeletal development in conditions of reduced movement. Advantages of the chick system include ease of access to the embryo for observation and manipulation. It enables multiple observations of the same embryo over the course of development, as chick eggs can be windowed for observation or cultured *ex ovo*.[26] Movement is most commonly reduced in chicks using neuromuscular blocking agents to induce rigid or flaccid paralysis *in ovo*, or skeletal rudiments can be cultured and manipulated *in vitro*.[27-35] Pharmacological agents such as the neurotransmitter-blocker curare,[36] can also be used in pregnant rodents to block foetal movement. However, administration of pharmacological agents is intrusive and is more technically demanding. In contrast, the mouse offers the possibility of more sophisticated genetic manipulation. Mutant embryos where specific genes required for muscle formation and contraction have been inactivated provide more convenient and absolute models of immobilisation. Different genetic mutations result in reduced, absent, or non-contractile skeletal muscle,[37-41] allowing examination of skeletal effects in a variety of environments. Reduced muscle and immobilisation models have also been developed in zebrafish,[42-44] whose transparent embryos allow for easy visualisation and live imaging of development.

An overview of normal skeletal development

Limbs first appear as small buds of mesenchymal cells covered by ectoderm at species-specific positions along the anteroposterior (AP) body axis[20,21,45] (Fig. 1 (1)) The first sign of skeletal development is the condensation of mesenchymal cells at the core of the bud[46] in the location of future skeletal elements (Fig. 1 (2)). In the proximal forelimb bud for example, a y-shaped condensation represents the future humerus, radius, and ulna. Distal condensations are progressively added and future joint sites become apparent as areas of increased cell density called interzones (Fig. 1 (2)).[47,48] Mesenchymal condensations prefigure an immediately subsequent pattern of cartilage differentiation (chondrogenesis), indicated first by expression of the Sox9 transcription factor[49] (Fig. 1 (3), Fig. 2 (i)). The emerging cartilage cells (chondrocytes) build up an extracellular matrix (ECM) of collagens, primarily types I and II, and structural proteoglycans in an avascular environment.[50-53] This transient cartilage template is progressively replaced by bone via endochondral ossification (Fig. 2 (ii, iii)). At the joint interzones (Fig. 1 (2), Fig. 3), cells are organised into three territories which later form the permanent articular cartilage at the ends (epiphyses) of adjacent rudiments, and the intervening joint cavity, encapsulated by a synovial membrane. Thus,

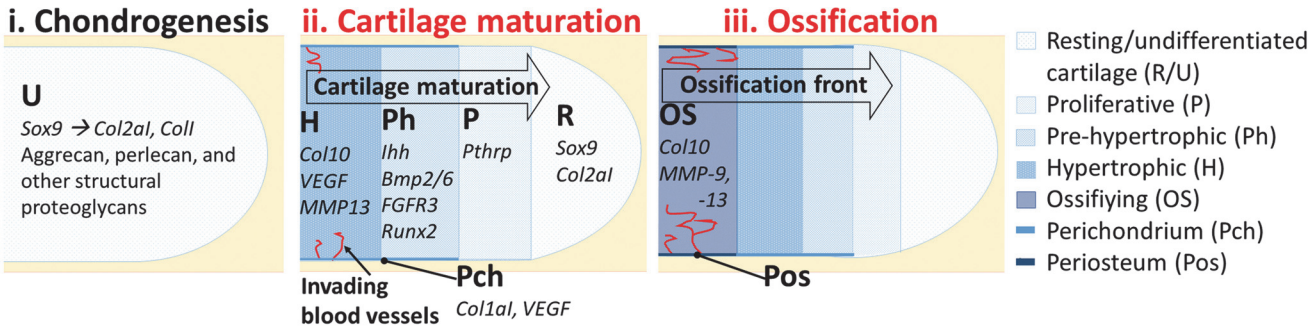

Fig. 2

Diagrams showing an overview of endochondral ossification. The initiation of cartilage formation (chondrogenesis) is marked by expression of the Sox9 transcription factor, followed by production of an extracellular matrix (ECM) of collagens I and II and structural proteoglycans (i). This transient cartilage matures progressively. Cartilage cells (chondrocytes) progress through stages of proliferation, pre-hypertrophy, and hypertrophy (ii). Maturation commences at the mid-point of the long bone shaft and proceeds toward the ends of the long bone. Each stage is marked by expression of a particular cohort of genes (examples indicated). As chondrocytes undergo hypertrophy and die, they are replaced by bone-forming cells (osteoblasts), carried in from the perichondrium via invading blood vessels (red) (iii). Osteoblasts employ matrix metalloproteinases (MMPs) to break down the cartilage ECM and replace it with collagen X-rich bone. Processes affected by immobilisation are denoted in red (explained in the text).

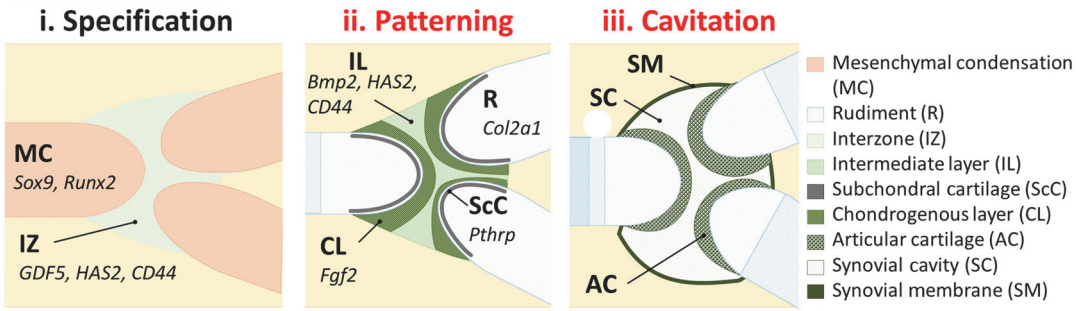

Fig. 3

Diagrams showing an overview of synovial joint formation. Future joint locations are first identifiable as regions of increased cell density, the joint interzones (i). At these interzones, cells organise into morphologically and molecularly recognisable zones. The chondrogenous layers are distinct from the intermediate layer, each territory expressing specific genes (examples shown) (ii). Permanent articular cartilage forms at each of the rudiment ends (epiphyses), and the joint cavity, encapsulated by a synovial membrane, forms at the intermediate site (iii). Processes affected by immobilisation are denoted in red (explained in the text).

formation of the limb skeleton involves co-ordinated endochondral ossification, differentiation of permanent cartilage at the joint interfaces, and construction of functional cavitated joints, in addition to the development of associated structures such as tendons, ligaments, and menisci.

The well organised spatial patterns of cell differentiation in the developing limb build over time, with initial patterning established by molecular signalling from the adjacent ectoderm and flank mesoderm, influencing patterns of chondrogenesis as the limb buds grow out.[54,55] Our knowledge of such mechanisms is advancing steadily. For example, a Turing reaction-diffusion mechanism involving an integrated wingless-related integration site (Wnt) and bone morphogenetic protein (BMP) signalling and the key chondrogenic transcriptional regulator Sox9, has recently been demonstrated to generate the multiple digit pattern in the distal limb.[56] Such early patterning mechanisms can set up crude morphogenically-determined spatial territories which are then developed with local cell–cell interactions and signalling, as described below.

During endochondral ossification (Fig. 2), the chondrocytes transition through proliferative, pre-hypertrophic and hypertrophic phases, apoptosis, and are replaced by bone-forming cells (osteoblasts) at the ossification site, which begins at the mid-point of the diaphysis and spreads to both epiphyses. The perichondrium encapsulates the rudiment and regulates hypertrophy of adjacent chondrocytes.[57] It also contributes to ossification by generating osteoblasts which build the periosteum (bone collar) locally and are carried into the cartilage rudiment by invading blood vessels to ossify the hypertrophic zone.[58,59] As the chondrocytes mature, the ECM is also remodelled. Matrix metalloproteinases assist in degradation of the collagen II- and aggrecan-rich ECM of immature chondrocytes, allowing for hypertrophic chondrocytes to build a collagen X- and matrix glycoprotein-rich environment to be mineralised by invading osteoblasts.[60-63] At birth, most of the cartilage

template is replaced by mineralised bone with the exception of growth plates, which will persist as a source of immature chondrocytes for elongation of the long bone until the organism reaches its adult size. Permanent articular cartilage persists at the ends of the long bones at the point where it caps the epiphyses to reduce friction at joint articulations.

Mature synovial joints of the limb allow for a wide range of movement. This type of joint consists of bones separated by a fluid-filled cavity, encapsulated by a double membrane.[64] Synovial joint formation can be divided into three distinct stages:[65] definition of the joint site (specification) (Fig. 3 (i)) is followed by differentiation of joint cell territories (patterning) (Fig. 3 (ii)), and finally formation of the joint cavity (cavitation) (Fig. 3 (iii)). Morphogenesis of the rudiment ends occurs as tissue territories are defined within the joint prior to cavitation.[66] The molecular programme responsible for joint specification is largely unknown, however, recent efforts have identified a target gene of the non-canonical transforming growth factor-beta (TGF-β) pathway, c-Jun, as an early determiner of synovial joint position,[67] influencing expression of the well-established early molecular marker of the joint interzone, Gdf5.[68]

Three distinct cellular layers can be distinguished within the interzone based on cell density, orientation, and gene expression. The joint cavity itself forms at the midline (intermediate layer), while the two outer chondrogenous layers contribute to articular cartilage.[69] Genetic lineage tracking experiments have proved useful for determining interzonal cell origin. Articular and epiphyseal plate chondrocytes were shown to have a common Sox9-positive identity during early chondrogenesis,[70] however, these cells diverge into distinct populations of interzone and transient cartilage cells from the earliest discernible stage of joint development. Gdf5-positive interzone cells give rise to articular cartilage, ligaments, and the lining of the synovium,[69] while matrilin-1-positive non-articular chondrocytes never contribute to the joint.[71] The conditions which lead interzonal cells to differentiate into persistent cartilage, rather than proceed through hypertrophy and ossification, are not fully understood; yet, some molecular clues exist. For example, cells in permanent cartilage never express pre-hypertrophic markers such as Indian hedgehog (Ihh) and BMP6.[72]

There is also a contribution of cells migrating into the intermediate zone of the joint at later stages (E14.5) from surrounding non-cartilaginous tissues,[65,73] and cells lying posterior to the nascent elbow joint have been shown to migrate and contribute to joint regeneration following surgical removal in the chick embryo.[74] TGF-β type II receptor expressing cells reside at the periphery of the joint until later in development (E16.5) when they contribute to joint structures such as the synovial lining, ligaments, and menisci.[75] Peripheral joint cells such as these could be slow-cycling stem/progenitor cells. It is unknown how these cells might relate to stem cells isolated from mature articular cartilage.[76]

Joint specification and tissue patterning are followed by cavitation, where the midline of the interzone transitions from a cell-dense region to a cell-free fluid space. Key to this transition is localised production of hyaluronan, a diffuse ligand found at the interzone of developing joints and in the lubricating synovial fluid of mature joints.[77,78] A transition from cell cohesion to dissociation caused by the increased production of hyaluronan may be responsible for joint cavitation.[79,80]

The effects of reduced embryo movement on endochondral ossification, rudiment morphology and joint formation

The thin, hypomineralised bones of infants subject to reduced foetal movement indicate that immobilisation affects the process of bone formation.[11] Animal models of embryonic immobilisation confirm that endochondral ossification and joint formation are profoundly affected by altered movement (indicated in Figures 2 and 3, respectively; also described in supplementary Table i). In both chick and mouse models, pharmacologically- or genetically-induced immobilisation causes abnormal ossification[81,82] and mechanically substandard bones.[83] Reduced ossification of tibiotarsi in neuromuscular-blocked chick embryos was accompanied by altered expression of chondrocyte maturation markers Ihh and type X, alpha 1 collagen (Col10a1) in pre-hypertrophic and hypertrophic zones, respectively, suggesting reduced proliferation at the expense of maturation.[31] A similar effect was seen in muscleless mouse embryos with reduced and misshapen ossification fronts in some rudiments, in particular the humerus and scapula. Interestingly, forelimbs and proximal rudiments were more affected than hind limbs and distal rudiments, respectively.[82] Although different molecular cues present in fore- and hind limbs may contribute to this phenomenon,[84] fore- and hind limbs could in fact experience different sources and combinations of mechanical stimulation. Computational modelling predicted that hind limbs experience more stimulation through passive movement (displacement caused by maternal and littermate activity), which might partially compensate for the lack of intrinsic movement.[85] This finding, combined with the transient nature of TBBD in infants, suggests that controlled physical therapy has the potential to alleviate the clinical consequences of reduced embryonic movement.

Growth and maturation of embryonic skeletal rudiments are intrinsically linked processes and immobilisation has been shown to affect size and shape by altering the balance of these cellular events. Immobilised mouse and chick embryos demonstrate cartilaginous rudiments which are shorter and have poorly-defined

morphological features such as condyles or tuberosities,[30-33,82,86-88] corresponding to reduced chondrocyte proliferation in the growth plate,[89] terminal condyles,[90] and bony eminence of the humeral tuberosity[91] under conditions of immobilisation or altered mechanical input. *In vitro*, proliferation of immature chondrocytes is stimulated by cycles of mechanical stress,[92] suggesting that immobilisation during embryonic development could inhibit chondrocyte proliferation in diverse regions of skeletal growth, thereby altering the size and morphology of rudiments and their ultimate functionality. Another aspect of cartilage morphogenesis shown to be disrupted in immobilised zebrafish and mildly affected in mice is the intercalation of chondrocytes into columns organised along the elongating rudiment.[43]

The occurrence of hip dysplasia following reduced movement in human infants indicates an effect on joint formation[14] and early studies on immobilised chick embryos showed dramatic fusion of joints in some cases.[93] Now a variety of studies in animal models have shown joint reduction or fusion in limbs as well as vertebrae and head joints.[27-30,32,82,86,87,94-99] Although most chick studies have used rigid paralysis, flaccid paralysis also causes joint reduction,[28] and hypermobilisation results in joint enlargement.[100] These findings collectively demonstrate that appropriate dynamic stimulation is necessary for synovial joint formation.

As outlined earlier, joint development involves a number of phases (Fig. 3) and immobilisation studies have begun to reveal which of these are influenced by movement. Specification is not affected since joint sites, marked by cells expressing Gdf5, appear correctly positioned in immobilised embryos. However, specific joint tissues fail to differentiate appropriately at the joint sites.[32,82,99,101] In chicks, immobilisation causes notable joint tissue disorganisation before cavitation: tissue-specific expression of fibroblast growth factor 2 (Fgf2) in the chondrogenous layer and BMP2 in the intermediate layer is lost, while rudiment-specific type II, alpha 1 collagen (Col2aI) and parathyroid hormone-related protein (Pthrp) are expressed across the joint territory,[32] which is similar to findings in muscleless mouse embryos.[82] These findings show that signalling events which delineate between tissues at developing joints are seriously affected by altered mechanical input, as specified interzone cells lose their joint identity and instead adopt the chondrogenic fate of their neighbours. Joint morphogenesis precedes cavitation[102] and, as previously mentioned, is also impacted by immobilisation with alterations to the shape of condyles in immobilised chick embryos.[32] Movement therefore contributes to a mechanically-stimulated gene regulatory programme responsible for appropriate rudiment morphogenesis and joint differentiation before cavitation in order to determine synovial joint functionality.

A mechanical basis for the impact of movement: the integration of molecular and biophysical signalling

The mechanical environment. As cells proliferate and differentiate into dedicated tissues, they respond to spatially- and temporally-organised signals including mechanical forces. Skeletal movement delivers mechanical loading in the form of several types of specific physical stimuli such as stress, strain, hydrostatic pressure and fluid flow.[81,90] The stimuli generated in developing tissues cannot currently be directly measured, however, finite element (FE) analysis, informed by the morphology of emerging tissues in the limb and direct measurement of mechanical properties of the developing interzone, can predict patterns of stimuli generated across space and time by limb flexion/extension. This modelling shows that dynamic patterns of predicted stimuli correspond spatially and temporally to those tissues and processes most affected by reduced movement.[66,81,85,90] For example, peak stimuli at the mid-diaphysis before the onset of ossification spread proximally and distally ahead of the ossification front.[81] In the knee joint, FE models predict peak stimuli at the future patella and condyles of the distal femur, as well as dynamic patterns of hydrostatic pressure among the emerging territories of the joint interzone. All of these elements are affected by immobilisation.[32] These findings confirm that mechanical forces in the developing skeleton are dynamic and linked to the processes of tissue patterning and maturation.

Efforts to recapitulate developing joint or bone tissue formation *in vitro* have used mesenchymal stem cells (MSCs) as multi-potent progenitor cells, as they are capable of forming fat, cartilage and bone under appropriate culture conditions.[103] As in embryonic tissues, mechanical stimuli have profound effects on the differentiation of stem cells in culture; cyclic pressures at varying magnitudes can induce chondrogenesis or osteogenesis when supplemented with appropriate biochemical signals.[104,105] Efforts to recapitulate cartilage and bone tissue formation also make use of adult-derived, induced pluripotency stem (iPS) cells.[106,107] These cells are self-renewing and respond to biochemical signals for cartilage and bone formation, indicating that they may be useful for tissue engineering regimes which incorporate mechanical stimulation.

The molecular environment. During endochondral ossification and joint development, a balance of factors affecting multiple signalling pathways, including transforming growth factor beta (TFG-β)/BMP, FGF and Wnt, calibrates an appropriate rate of cartilage maturation.[108-111] As a result, distinct regions of the developing endochondral skeleton have characteristic gene expression profiles. Parathyroid hormone-related protein (Pthrp) marks proliferating chondrocytes, while Ihh signalling in pre-hypertrophic chondrocytes spurs on maturation. A feedback loop of these factors

guarantees a steady rate of maturation.[112-114] Additional signals are also exchanged between the perichondrium and the enclosed cartilage, indicative of a mutually-dependent relationship between these two structures.[115,116] Furthermore, hedgehog signalling is also implicated in multiple steps of joint development,[117,118] and co-ordinated signalling between the two processes is clear.

Wnt signalling. In both bone and joint development, Wnt signalling is particularly complex. Spatial and temporal control of canonical (β-catenin-based) and non-canonical Wnt signalling is crucial to the correct differentiation of cartilage and bone in both the rudiment and joint. While chondrogenic differentiation in the rudiments requires suppression of the canonical Wnt pathway, canonical Wnt signalling is implicated in chondrocyte hypertrophy and osteoblastogenesis and inhibits proliferation.[110,119-121] Canonical Wnt signalling is also active at sites of joint formation, where it inhibits cartilage formation.[122-125] In contrast, the non-canonical Wnt planar cell polarity (PCP) and calcium (Ca^{2+}) pathways promote chondrogenesis.[120] Of note, activated PCP signalling is necessary for convergent extension of proliferative chondrocytes, which contributes to rudiment morphology.[126,127] Later, non-canonical pathways become important for osteoblastogenesis, and may be involved in crosstalk with the canonical Wnt and BMP pathways during bone formation.[128-130]

TGF-β/BMP signalling. In the cartilage rudiment, canonical TGF-β signalling expands mesenchymal cell populations, primes cells for chondrogenic signals,[131-133] encourages chondrocyte proliferation and acts against maturation and differentiation.[134-138] As noted earlier, TGF-β type II receptor is required at the joint and might support a joint-specific source of stem cells.[75] The BMP subfamily is present throughout the cartilage rudiment and in addition to influencing cartilage formation, it stimulates proliferation and alternately slows or accelerates hypertrophy in maturing chondrocytes both *in vivo* and *in vitro*.[58,139-145] BMP ligands, including Gdf5, and antagonists are expressed at developing synovial joints, suggesting that a balance of BMP signalling here is necessary to prevent overgrowth of chondrogenous tissues.[68,146,147] In addition to the canonical pathways involving Smad transcription factors, BMPs and other TGF-β superfamily ligands have also been shown to induce non-canonical mitogen-activated protein kinase (MAPK) signalling cascades both *in vivo* and *in vitro*.[148] Crosstalk between the canonical and non-canonical pathways can modulate chondrocyte differentiation and cartilage matrix synthesis.[149]

FGF signalling. FGF ligands and receptors (FGFRs) are present throughout limb bud development and across different zones of chondrocyte maturation, and take part in chondrogenesis, regulation of cell proliferation, and osteoblastogenesis.[150-154] FGF signalling is also found at the perichondrium and the presumptive joint.[101,152]

Interpretation and integration of mechanical signals by the developing skeletal system

As evidence builds regarding the importance of mechanical stimulation from movement for specific aspects of skeletal development, it is necessary that we clarify the mechanisms that integrate mechanical and molecular signals. We currently cannot explain how the cells of the developing skeletal system can sense and transduce such mechanical stimuli into cell differentiation and tissue patterning outcomes, however, exciting clues are starting to appear on two fronts: first, how the size, shape, and stiffness of the substrate influences how a cell behaves and second, how developmentally important signalling pathways are primed or modulated by mechanical cues.

Wide interest was generated in the influence of mechanical cues on cell differentiation following the demonstration that stem cells are primed for differentiation by the stiffness of their substrate, with rigid, stiff, or soft substrates supporting osteogenic, myogenic, or neurogenic differentiation, respectively.[155] This is sensed through integrin receptors coupled to the cytoskeleton and controlled by the Ras-homolog gene family, member A/Rho-associated, coiled-coil containing protein kinase (RhoA/ROCK) pathway which moderates intracellular signalling and is implicated in both chondrogenesis and osteogenesis.[156-159] Elegant experiments have shown that cell shape alone, reflective of substrate stiffness, can influence cell differentiation.[160,161] Through the physical framework of the cell, the extracellular environment is tethered to the nucleus, meaning that any change in the stiffness of the ECM or deformation of the cell can influence cell behaviour.

It has also been established that a cell can sense its micro-enviroment through primary cilia, present on most cell types.[162] These cilia extend into the ECM and can sense mechanical forces such as the bending induced by fluid flow.[163] Stimulation of the primary cilium can influence how the cell responds to Ihh, Wnt, and TGFβ signalling[164-166] and may have wider effects on other signalling pathways.[165] Primary cilia have been detected in the developing skeleton, and their function is necessary for endochondral ossification.[167,168] They can also influence the fate of cultured MSCs,[169] which suggests a prominent role for sensory cilia in mechanotransduction during tissue patterning and cell fate determination in the developing skeleton.

As described here, the physical state and environment of the cell can influence its transcriptional activity and differentiation, but so too can exposure to signalling pathway ligands, together with known agonists and antagonists. The cytoskeleton is a highly dynamic system which is interlinked with cell signalling processes and could offer a link between mechanical environmental cues and a chemical cellular response within the developing skeletal system.[170] For example, the Wnt and TGF-β pathways regulate the actin cytoskeleton and, in turn,

these pathways and others are affected by cytoskeletal dynamics.[171-174] In immobilised limbs, numerous genes related to cytoskeletal support and architecture are regulated differentially.[175] Changes in cytoskeletal architecture in muscleless mice, for example, could affect the transport of intracellular components, which could in turn mediate or amplify alterations in cell signalling to affect cell differentiation.

The ECM of cartilage consists of collagen fibres and glycoproteins, and provides another means to facilitate the mechanosensory reactions of chondrocytes. Collagens provide structure to the cartilage, as do glycoproteins, which protect the tissue against compression and mechanical stress and can differentially regulate the diffusion and binding of signalling.[176] Maturation of chondrocytes is accompanied and, perhaps, regulated by changes in ECM composition during endochondral ossification.[62] Focal adhesions mediate cell–matrix interactions via integrins, which link extracellular matrix components to intracellular actin filaments. In addition to physical linkers, the complex contains the signalling molecules which control focal adhesion turnover and breakdown of the cell–matrix connection. Proper cell–matrix junctions are necessary for chondrocyte differentiation.[177]

Integrins and ion channels are other potentially mechanosensitive components which could transduce physical pressures to biochemical intracellular signals to regulate growth and development of chondrocytes.[178-180] Blocking integrins and stretch-activated ion channels inhibits downstream signalling and cellular proliferation in chondrocytes.[92,181] Additionally, mechanical loading can alter integrin expression or matrix binding through the release of soluble signalling molecules.[182]

Wnt signalling and mechanical stimulation. We recently highlighted the potential role of the Wnt signalling pathway in mechanotransduction in the developing skeletal rudiment.[175] Comparison of the transcriptomes in muscleless, compared with control, mouse humeri at TS23, when ossification commences and the skeletal phenotype is first recorded, shows enrichment for genes associated with cytoskeletal architecture, cell signalling, and development and differentiation. Alteration of these biological processes in muscleless mouse skeletal rudiments hints at a relationship between cytoskeletal function and cell signalling during patterning of skeletal tissues. Furthermore, among the multiple cell signalling pathways affected in muscleless embryos, the greatest number of differentially regulated genes are associated with the Wnt pathway, and alteration in the spatial expression of several key pathway components was also documented, further suggesting that disruption of Wnt signalling could be responsible for abnormal skeletal patterning during mechanical inhibition.[175]

As described earlier, the Wnt pathway is well documented as an important driver of embryonic patterning and development. However, its integration with mechanostimulation *in vivo*[99,175,183-185] and *in vitro*[184-187] via both direct and downstream regulation of other signalling cascades, opens a new avenue of exploration for this important regulatory system.

Potential therapies for skeletal developmental defects and skeletal regeneration

The vertebrate musculoskeletal system has evolved to construct functional and adaptable components, using available molecular and biophysical cues. Unravelling the impact of mechanical stimulation generated by embryonic movement will advance our fundamental understanding of skeletal development which will reveal in particular how the system integrates multiple types of information at the cellular level. Furthermore, discoveries in this area have evident biomedical implications and could lead to the development of novel therapeutics to inform treatments for restricted foetal movement *in utero* and also for age- or injury-related degeneration of skeletal tissues.

In the case of congenital problems caused by reduced movement such as low bone mineral density or joint dysplasia, a better understanding of the type and magnitude of stimulation required for normal skeletogenesis could lead to the development of appropriate physical therapy regimes *in utero* to substitute for autogenerated movement.[85] Post-natal supplemented diets do not seem to be effective enough to reverse low BMD that is already present,[188] suggesting a need for movement-based therapies. This is encouraged by the success of gentle yet effective exercise programmes to treat reduced BMD in premature babies.[189-191] This represents a simple but meaningful method for caregivers and parents to ensure the physical fitness of affected newborns, with the potential for lifelong results.[192] Another possibility for therapeutic intervention is treatment with ultrasound, which is suggested to mimic the effect of mechanical loading on bone formation.[193] Although treatment for joint fusion can be quite invasive, a combination of surgical treatment and physical therapy, started during infancy, can reduce lifelong impediments to movement in individuals with severe contractures.[194,195]

Mechanical forces are not just important during development. Strong bones and smoothly-articulating joints are interrelated features of a healthy skeleton and facilitate movement and an active life. Biophysical cues generated by movement in turn feed back to maintain and remodel skeletal tissues throughout life.[196,197] With age, we not only become less mobile, but also our skeletal tissues become less responsive to loading.[198] Although changes in the hormonal environment are certainly important in the reduced response and onset of osteoporosis,[199-201] the current emphasis on hormonal replacement treatment could be augmented by uncovering the correct stimulation required to shift the balance between resorption and deposition of bone, and thus enhance skeletal integrity.[202]

Appropriate and effective administration of therapeutic regimes to affected individuals will require a precise understanding of both molecular and mechanical controls of skeletogenesis, as well as the complex interplay between these types of cues during the development and maintenance of healthy bones and joints. As mice and chicks are particularly useful for investigating the effect of mechanical stimulation on skeletal patterning, signalling and cellular events in these models are currently being explored with the aspiration of applying such findings to therapeutics in humans. The discovery that appropriate mechanical signals are instrumental in guiding correct differentiation of permanent articular cartilage is of particular importance in informing regimes for the generation of articular cartilage from stem cells for joint replacement therapies. Currently, adult-derived stem cells cannot be induced to form permanent cartilage *in vitro,* but instead proceed to hypertrophy, and therefore cannot be used for replacement of articular cartilage, necessitating prosthetic replacement of the joint in the case of osteoarthritis and joint injury. Understanding how dynamic mechanical stimuli guide the formation of stable articular cartilage in the embryo would have an enormous potential benefit for the establishment of protocols to guide stem cell differentiation and in developing less invasive and more sustainable therapies in such cases. We still have much to learn about how cells integrate molecular and mechanical signals to form particular cell types stably, and how this knowledge can be translated clinically. Nevertheless, research in this area holds great promise for future practical applications.

Supplementary material

A table showing the observed effects of reduced or absent movement on skeletal development is available alongside the online version of this article at www.bjr.boneandjoint.org.uk

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- None declared

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