Building a co-ordinated musculoskeletal system: the plasticity of the developing skeleton in response to muscle contractions

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ABSTRACT

The skeletal musculature and the cartilage, bone and other connective tissues of the skeleton are intimately co-ordinated. The shape, size and structure of each bone in the body is sculpted through dynamic physical stimuli generated by muscle contraction, from early development, with onset of the first embryo movements, and through repair and remodelling in later life. The importance of muscle movement during development is shown by congenital abnormalities where infants that experience reduced movement in the uterus present a sequence of skeletal issues including temporary brittle bones and joint dysplasia. A variety of animal models, utilising different immobilisation scenarios, have demonstrated the precise timing and events that are dependent on mechanical stimulation from movement. This chapter lays out the evidence for skeletal system dependence on muscle movement, gleaned largely from mouse and chick immobilised embryos, showing the many aspects of skeletal development affected. Effects are seen in joint development, ossification, the size and shape of skeletal rudiments and tendons, including compromised mechanical function. The enormous plasticity of the skeletal system in response to muscle contraction is a key factor in building a responsive, functional system. Insights from this work have implications for our understanding of morphological evolution, particularly the challenging concept of emergence of new structures. It is also providing insight for the potential of physical therapy for infants suffering the effects of reduced uterine movement and is enhancing our understanding of the cellular and molecular mechanisms involved in skeletal tissue differentiation, with potential for informing regenerative therapies.

1. INTERDEPENDENCE OF THE MUSCULATURE AND THE SKELETON: THE IMPORTANCE OF MECHANICAL REGULATION

The musculature and the skeleton are intimately linked throughout life, from development, through homeostasis and aging. In his seminal work in 1917, *On Growth and Form*, D'Arcy Thompson wrote that "between muscle and bone there can be no change in the one but it is correlated with changes in the other"(Thompson 1917). This relationship has long been recognised in the remodelling of bone in response to physical stimulation (e.g. increased bone density in the dominant limbs of athletes), reduced stimulation (e.g. space flight) and the loss of mechanoresponsiveness in degenerative skeletal diseases such as osteoarthritis and osteoporeosis (Vico and Hargens 2018; Ozcivici et al. 2010). In 1888, Wolff's law identified bone strength as being proportional to physical loading and was incorporated by Frost in a "mechanostat" theory of bone as an adaptable material where biochemical changes within the tissue, that carry out bone remodelling, are responsive to forces exerted by the muscle (Frost 2001; Chen et al. 2010). Furthermore the interdependency works both ways with muscle pattern also responding to skeletal morphology (Tokita and Schneider 2009).

Functional muscle is not only important for healthy maintenance of the skeletal system but muscle derived mechanical forces generated by embryo movements are required for development of a healthy skeleton, as evidenced by human congenital abnormalities in cases of reduced foetal movement (reviewed in (Shea et al. 2015). In 1983 Moessinger formalised a description of Foetal Akenesia Deformation Sequence (FADS) including multiple joint contractures, facial abnormalities, foetal growth retardation and short umbilical cords (also known as Pena and Shokeir phenotype)(Hall 2009; Moessinger 1983). Noting the similarity of effects seen in a rat model where a muscle relaxant was administered to pregnant females, he pointed out that despite varied aetiologies the common factor in theses congenital cases is reduced foetal movement. Some causes of reduced movement are intrinsic to the foetus, including neuromuscular disorders, while others are extrinsic, for example due to abnormal uterine structure or multiple foetuses leading to movement restrictions. With extrinsic causes there is generally a better chance of survival and recovery with postnatal physiotherapy (Niles et al. 2019). Cases of multiple joint contractures have also been referred to as arthrogryposis multiplex congenita (AMC) with 10% of such cases also showing hypomineralised and fragile long bones, prone to fracture at birth (Hall 2014; Niles et al. 2019; Rodríguez and Palacios 1991; Miller and Hangartner 1999). This temporary brittle bone disease is distinct from *osteogenesis imperfecta* (Forlino et al. 2011), permanent brittle bone disease, caused by mutations in collagen genes, and its regression, usually within the first year of life, shows the plasticity of the mechanical response of the developing skeleton. Joint dysplasia, in particular Developmental Dysplasia of the Hip (DDH), is more common in new born infants (reviewed in (Nowlan 2015) and has been associated with abnormal positioning of the femoral head in the acetabulum, due to abnormal limb position, uterine pressure or ligament laxity (Shefelbine and Carter 2004).

As early as the 1930s animal models were used to show the effects of embryo immobilisation on the developing skeleton (e.g. (Murray and Selby 1930; Fell and Canti 1934) but a more concerted focus in recent years, using a variety of immobilisation approaches and different species (Table 1), has deepened appreciation for the role of muscle in directing appropriate skeletal formation, and is starting to provide important insights into the cellular and molecular mechanisms involved. Understanding how mechanical stimulation from muscle influences cell differentiation and tissue patterning during development of skeletal tissues is of particular interest as uncovering the

mechanisms involved holds promise for biomedical application for improving tissue regeneration and successful tissue engineering.

Interdependency between functional muscle and skeleton ensures the maintenance of a functional system for locomotion even if one or other tissue is altered, but the mechanical response during development of the skeletal system is also a striking example of developmental plasticity (West-Eberhard 2003). This contributes to our understanding of adaptive evolution through morphological integration (Hallgrímsson et al. 2002) providing an explanation for the emergence of new co-ordinated morphologies as seen in the huge variety of vertebrate limb structures from human arms and hands to bat wings and dolphin flippers.

The musculoskeletal system is made up of interconnected muscles, bones and cartilages with other connective tissues surrounding the muscle, attaching it to bone (tendons) and interconnecting bones (ligaments). Different embryonic origins of cells form the skeletal elements in different parts of the embryo e.g. lateral plate mesoderm at the level of the limbs gives rise to the limb skeleton and migrating neural crest cells derived from the neural ectoderm contribute largely to the cranial skeleton. Bones can be formed by two entirely different processes: endochondral and intramembraneous ossification. Despite the diversity of processes involved there are examples of mechanoresponsiveness to muscle forces throughout the skeletal system. Here we present examples of the importance of biophysical stimuli from muscle contraction for multiple aspects of bone, joint and tendon formation, with particular focus on the limb skeleton, the secondary cartilages of the jaw and clavicle, and the formation of the spine. We further review current evidence that reveals aspects of the cellular and molecular mechanisms involved and the important gaps still to be filled.

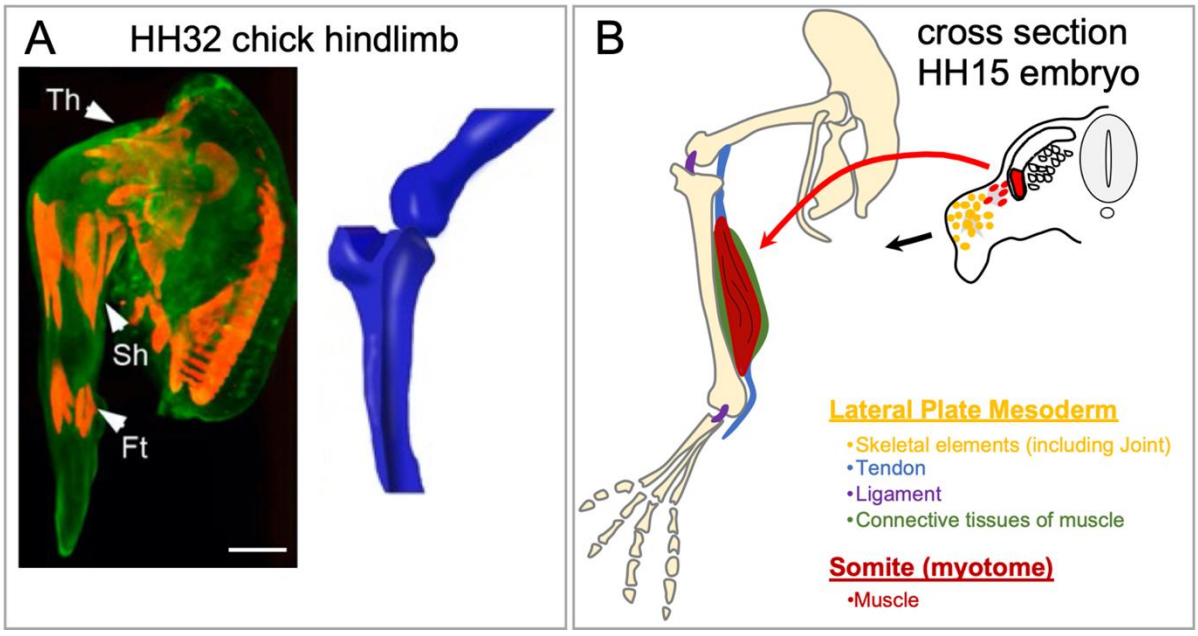

Figure 1: Co-ordinated development of muscle, tendon and the cartilage template of future bones in the developing chick embryo limb. A) chick hindlimb at Hamburger and Hamilton (HH) stage 32 (Hamburger and Hamilton 1992) showing developing tendons in green (α-Tenascin antibody) and developing muscle masses in red (α-myosin antibody); 3D representation of developing cartilage rudiments in blue. (Adapted from (Roddy et al. 2009). Th indicates thigh musculature, Sh; shin; Ft; foot. B) Diagrammatic representation of the developing musculoskeletal system in the embryonic

chick hindlimb including indication of the different embryonic origins of muscle (somite) and skeleton, tendons, ligaments and other connective tissues (lateral plate mesoderm) in the earlier embryo. Diagram on the right shows a cross section through the limb bud region of a HH15 embryo indicating the cellular origins of muscle and skeletal cell types, as indicated. Scale bar 1mm

2. INTERDEPENDENCE OF MUSCLE AND SKELETAL TISSUES IN THE DEVELOPING VERTEBRATE LIMB: THE MECHANICAL RESPONSE

Vertebrate limbs arise as buds of mesenchyme cells covered in ectoderm along the flank of the embryo. These buds elongate and subdivide into three territories along the future shoulder to digit tip (proximal to distal) axis, widening to a flattened paddle at the distal end to form the digital plate (autopod), while the more proximal regions give rise to the stylopod (where the humerus or femur will form) and the zeugopod (where the radius and ulna or tibia and fibula will form) (reviewed in (Zeller et al. 2009; McQueen and Towers 2020)). The component tissues of the limb have distinct embryonic origins: the cells that give rise to the skeletal rudiments, joints, tendons and the connective tissue surrounding the muscle originate from the lateral plate mesoderm at the site of the emerging limb bud while the future muscle cells migrate into the limb bud from more dorsally located somites (Christ et al. 1977; Chevallier et al. 1977) (Figure 1). Despite different origins there is striking co-ordination from earliest stages in the differentiation and patterning of the future muscle, tendons and skeletal rudiments (Kardon 1998; Roddy et al. 2009) (Figure 1). Cells condense at the core of the limb bud, switching on *Sox9* expression and differentiating as cartilage to form a template for the future skeletal rudiments, first visible as a Y shaped anlage prefiguring the most proximal elements (e.g. humerus, radius and ulna), with condensation and differentiation extending and branching progressively distally into the digital region (Thorogood and Hinchliffe 1975). The close relationship between the emerging tissues, not only allows trophic interactions but also mechanical input, most obviously resulting from the movement generated by embryonic muscle contractions.

Developing limbs experience a large range of visible movements compared to other regions of the embryo so it is not surprising that limbs provide some of the clearest examples of the importance of movement for appropriate skeletal development. Spontaneous muscle contractions begin early in the vertebrate embryo: In humans, first movements were recorded at nine weeks (approximately Carnegie stage 18)(de Vries and Fong 2006); similarly in the mouse model of mammalian development, clear embryo movement also begins just after the first muscle twitches at embryonic day (E) 12.5/Theiler stage (TS)20 (Theiler 1989) (Suzue and Shinoda 1999). In the chick we have recently refined earlier observations to reveal body movements from E3 /Hamburger and Hamilton (HH) stage 22 and the first limb displacements at HH23 (Rolfe et al. 2021; Hamburger and Balaban 1963) which reflects observed sensitivity to early immobilisations.

The mouse and chick provide complimentary models for embryonic immobilisation, each with valuable advantages. Access to chick embryos developing in externally laid eggs allows easy visualisation and immobilisation via surgery or addition of pharmacological agents; most commonly decamethonium bromide to induce rigid paralysis, pancuronium bromide for flacid paralysis and 4-aminpyridine or reserpine for hypermobility (Table 1; previously reviewed (Nowlan et al. 2010b)). In the mouse, several different genetic lines harbour mutations that affect muscle development and permit analysis of limb skeletal development in the complete absence of muscle (*Pax3*$^{Spd/Spd}$ (Vogan et al. 1993) and *Myf5*$^{nlacZ/nlacZ}$*:MyoD*$^{-/-}$ double mutants (Kablar et al. 2003)), with reduced muscle in *Myf5*$^{nlacZ/+}$

: *MyoD*$^{-/-}$ embryos (Rudnicki et al. 1993) and immobile muscle in muscular dysgenesis mouse embryos (*mdg/mdg*) (Pai 1965a) (Table 1). Figure 2 summarises the effects of immobilisation seen across mouse and chick models using different approaches to examine lack of movement.

Table 1: Most frequently used embryo immobilisation techniques

Mouse		
Genotype	**Effect**	**Example references**
Pax3$^{Spd/Spd}$ *(Splotch delayed)*	Muscleless limbs	(Vogan et al. 1993; Kahn et al. 2009; Rolfe et al. 2014; Shwartz et al. 2012; Shea and Murphy 2021)
Pax3$^{Sp/Sp}$*(Splotch)*	Muscleless limbs	(Franz et al. 1993; Nowlan et al. 2010a)
Myf5$^{nlacZ/nlacZ}$*:MyoD*$^{-/-}$	Muscleless	(Kablar et al. 2003; Rudnicki et al. 1993; Rot-Nikcevic et al. 2006; Nowlan et al. 2010a; Gomez et al. 2007; Sotiriou et al. 2019)
Myf5$^{nlacZ/+}$*:MyoD*$^{-/-}$	Reduced muscle	(Rudnicki et al. 1993; Nowlan et al. 2010a; Sotiriou et al. 2019)
Muscular dysgenesis (*mdg/mdg*)	Non-contractile muscle	(Pai 1965a; Kahn et al. 2009; Sharir et al. 2011)
Rat		
Treatment	**Effect**	**Example references**
Removal of amniotic fluid	Induced oligohydramnios	(Palacios et al. 1992)
Tubocurarine injection	Paralysis	(Rodríguez et al. 1992)
Chick		
Treatment	**Effect**	**Example references**
Decamethonium bromide (or decamethonium iodide)[1]	Rigid paralysis	(Drachman and Sokoloff 1966; Hall and Herring 1990; Mitrovic 1982; Persson 1983; Mikic et al. 2000; Hosseini and Hogg 1991a; Roddy et al. 2011b; Rolfe et al. 2021; Germiller and Goldstein 1997)
Pancuronium bromide	Flaccid paralysis	(Peterson et al. 2021; Osborne et al. 2002; Pitsillides 2006)
Reserpine	Hypermotility (low doses); paralysis (high doses)	(Ruano-Gil et al. 1985)
4-aminopyridine (4-AP)	Hypermobility	(Rolfe et al. 2021; Hammond et al. 2007; Pan et al. 2018)
Spinal resection - Surgical technique	Regional paralysis	(Drachman and Sokoloff 1966; Wong et al. 1993; Edom-Vovard et al. 2002)
Chorioallantoic graft or coelomic graft of early limb bud	No intrinsic movement	(Mitrovic 1982; Murray and Selby 1930; Hall 1986; Edom-Vovard et al. 2002)
Explant	No intrinsic movement (can be externally stimulated	(Lelkes 1958; Khatib et al. 2023)
Zebrafish		
Genotype/ treatment	**Effect**	**Example references**
myod^{fh261}	Most craniofacial muscles absent	(Hinits et al. 2011; Brunt et al. 2016)
MS222 anaesthetisation	Immobilisation	(Brunt et al. 2016)
myf5 and *myod* morphant	Absence of head musculature	(Hinits et al. 2009; Shwartz et al. 2012)
Nic mutant (b107)	Paralysis	(Westerfield et al. 1990; Shwartz et al. 2012)
0.016% tricaine anaesthetisation	Immobilisation	(Shwartz et al. 2012)

Note 1, other agents used: botulinum toxin (Murray and Drachman 1969; Drachman and Sokoloff 1966); succinylcholine (Ruano-Gil et al. 1978); (Hall 1972) and tubocurarine (Hall, 1972)

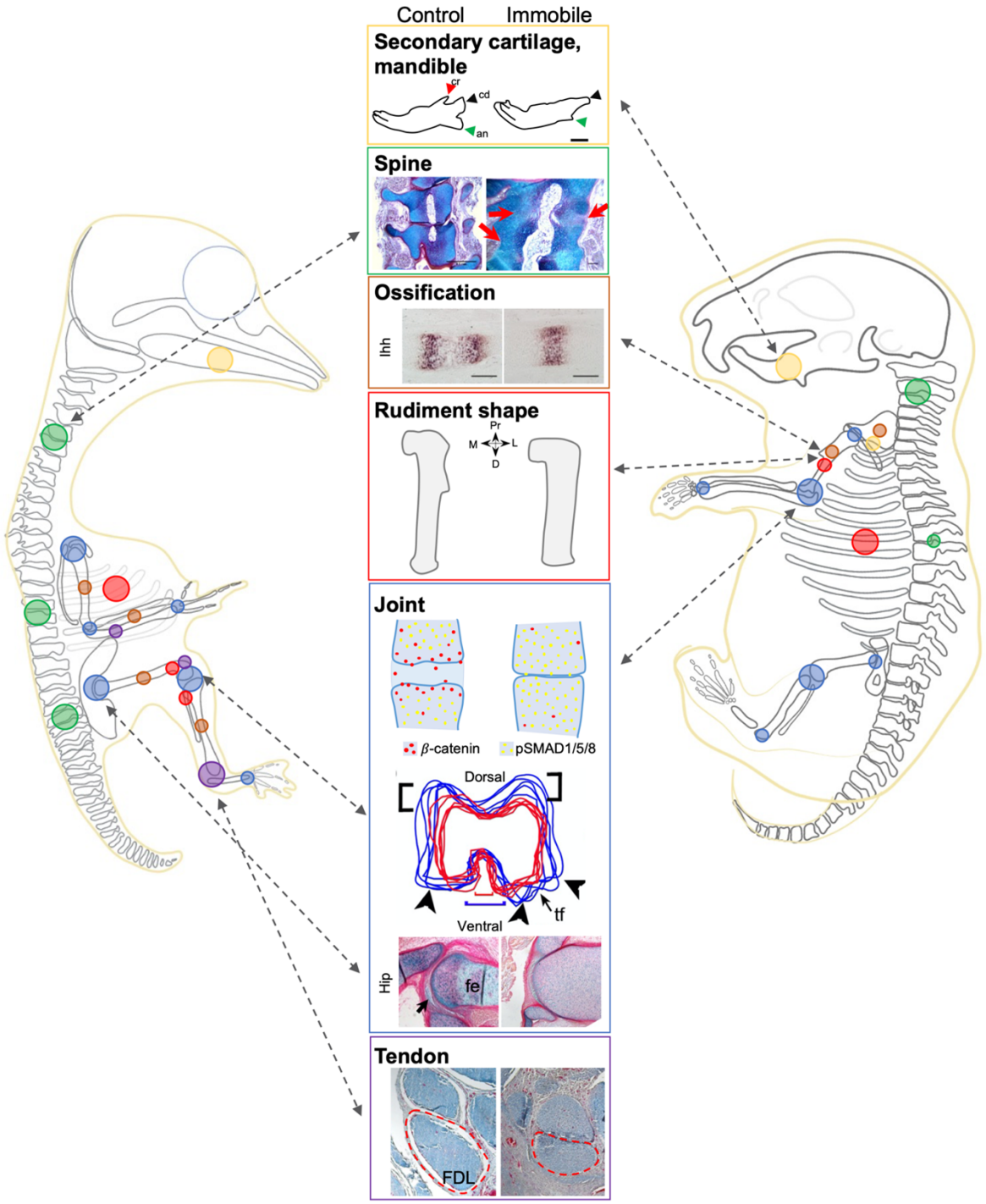

Figure 2: Overview of the main effects of immobilisation on the developing skeletal system in chick (left) and mouse (right) embryo experimental models, as reviewed here. Major effects are listed in the centre, with example illustrations. Approximate locations of recordings of these effects in the mouse and chick embryo are indicated by coloured circles using the colour key represented by the borders on the illustrations e.g. yellow; secondary cartilages, green; spine etc. **Secondary cartilage**: reduction/absence in mouse and chick mandible and mouse clavicle. Example shown is outline drawings of mouse mandibles showing reduced coronoid (cr), condylar (cd) and angular (an) processes in absence of muscle (Rot-Nikcevic et al. 2006). **Spine**: cartilaginous fusions (Red arrows) observed between adjacent cervical vertebrae following immobilisation. Scale bars 500 μm (control) and 100µm (immobile) (Rolfe et al. 2017). **Ossification**: delayed and abnormal ossification. The illustrated example shows sections of stage matched embryos with delayed expression of Ihh mRNA. **Rudiment**

shape: skeletal elements are shorter, more circular in circumference and smoother, lacking characteristic features. **Joints**: joint lines are reduced with fusion of cartilage across joints and abnormally shaped skeletal rudiment termini. Illustration of abnormal domains of Wnt and BMP signalling across an immobilised joint with cartilage fusion (Singh et al. 2018); outlines show contours of cross-sections through chick distal femoral condyles, control outlines in blue and immobilised outlines in red (Roddy et al. 2011b) (note in particular the flattened shape and reduced lateral condyle); example histology of hip joint (Rolfe et al. 2021). **Tendons**: late tendon development shows failure to mature with reduced size and spacing (Peterson et al. 2021). Effects not represented here include failure of sternal and palate fusion in immobilised embryos (Rot and Kablar 2013)

2.1 Effects of immobilisation on Joint formation

A key determinate of species-specific shape and structure in the vertebrate limb is the placement of synovial (diarthrotic) joints that separate the skeletal elements and allow full, frictionless motion (reviewed in (Rux et al. 2019)). Synovial joints also occur elsewhere, for example in the jaw. It has been well established that embryonic movement is required for the definition of appropriate tissue arrangement in synovial joints, providing clear evidence for the basis of the close relationship between emerging muscle, tendon, cartilage and bone in the limb and its importance for the formation of an integrated functional structure.

The first indication of the position of future joints is the appearance of a region of more flattened cells, the interzone, which goes on to form three distinct layers; the chondrogenic layers at the termini of the adjacent skeletal rudiments (future sites of articular cartilage), and an intermediate layer where the synovial cavity will form (reviewed in (Decker et al. 2014). Cell lineage studies show that interzone cells contribute to all joint tissues (Koyama et al. 2008), but the developmental process is dynamic with contribution of cells also from adjacent territories of transient cartilage and marginal cells (Ray et al. 2015; Shwartz et al. 2016). The emerging tissues go on to form the intricate structures that will facilitate smooth and stable movement; i.e. the complimentary shapes of the opposing articular cartilages, the stabilising structures including ligaments, joint capsule and lining surrounding the synovial cavity, filled with lubricating fluid.

The effect of embryo immobilisation on joint formation was first shown in early chick experiments following explant of the limb, surgical denervation or pharmacological immobilisation (Drachman and Sokoloff 1966; Fell and Canti 1934; Murray and Selby 1930; Ruano-Gil et al. 1978; Mitrovic 1982; Persson 1983; Lelkes 1958; Mikic et al. 2000; Roddy et al. 2011b), the common effects being loss of cavitation, fusion of multiple joints (limb and jaw synovial joints), and loss of menisci and patella. On the other hand, when hyperactivity was induced in chick embryos, joint cavities enlarged (Ruano-Gil et al. 1985). The muscle dysgenesis mouse (*mdg*), which has a spontaneous mutation leading to lack of muscle contraction and paralysis, was described as having abnormal joint cavitation (Pai 1965a). More systematic developmental analysis from early stages (E12.5) showed ectopic development of transient cartilage across the presumptive joint region, characterised by downregulation of joint specific markers and ectopic expression of an early marker of transient cartilage, *Col2a1*, with complete cartilage fusion in extreme cases (Nowlan et al. 2010a; Kahn et al. 2009; Roddy et al. 2011b; Rolfe et al. 2013) (Figure 2). While the earliest events of joint specification (interzone formation) were not affected, tissue patterning of emerging territories within the presumptive joint was lost.

It was particularly notable that while joint fusions were described at multiple joints including extreme effects at the elbow, the knee joint did not fuse in the mouse muscle-less and immobile muscle

embryos (Nowlan et al. 2010a; Kahn et al. 2009), although it did show loss of the patella (Eyal et al. 2015) and changes to the shape of the rudiment termini (Sotiriou et al. 2019). The apparent absence of effect on joint separation in the knee was puzzling since the knee joint is strongly affected in chick embryos (Roddy et al. 2011b). It was speculated that this may be due to the developing knee of muscle-less mouse embryos experiencing sufficient stimulation from passive movements from the uterine wall and littermates within the context of mammalian internal development (Nowlan et al. 2012) or, more recently, due to the acute flexion that occurs at the knee joint over time providing a threshold level of mechanical stimulation (Kim et al. 2022), rather than an intrinsic difference in the mechanosensitivity of mammalian knee joint developing tissues, which would be surprising. This has indeed been corroborated recently by mouse hindlimb explants being responsive to externally applied movement, forming more normal cavitation than unstimulated controls (Khatib et al. 2023).

Insight into the molecular mechanisms disturbed during joint formation has been achieved with the demonstration of loss of correct spatial localisation of Wnt and BMP signalling pathway activities at the joint line (Singh et al. 2018; Rolfe et al. 2018). The importance of Canonical Wnt/β-catenin signalling for joint formation was already recognised through mouse mutants (Guo et al. 2004; Später et al. 2006; Kan and Tabin 2013), but notably, immobilisation resulted in more extreme joint patterning disturbance and joint fusions than inhibiting Wnt signalling, indicating that additional molecular mechanisms are disturbed in the immobilisation effect (Rolfe et al. 2018). Kahn et al (Kahn et al. 2009) showed that Wnt signalling is disturbed in immobile mouse embryos and a prominent role for Wnt signalling was indicated by transcriptomic analysis comparing the humerus and associated joints of immobile embryos (*Pax3Sp^{d}/Pax3Sp^{d}*) and controls, which showed that Wnt was the most disturbed signalling pathway, with BMP signalling also highly disturbed (Rolfe et al. 2014). BMP signalling is normally active at a distance from the joint line, in territories of transient cartilage that will later be replaced by bone through endochondral ossification (Ray et al. 2015) while cartilage at the joint line will endure to form articular cartilage, so important for joint function. Therefore, taking advantage of different immobilisation approaches across mouse and chick, and the different advantages of the two models, we examined Wnt and BMP signalling activity at the joint lines of immobilised embryos compared to controls and showed that in immobile limbs, Wnt signalling activity is lost at the joint line while BMP signalling activity and *Col2a1* expression spreads across the abnormal joint territory (Singh et al. 2018) indicating that joint fusions are caused by the loss of appropriate spatial restriction of cell signalling defining the territories of transient and permanent articular cartilage (Figure 2).

The smooth movement and stability of the joint is dependent on complimentary, interlocking shapes of the interfacing ends of the bones; a feature also disturbed by immobilisation. Roddy et al (Roddy et al. 2011b) carried out shape analysis at the chick knee joint comparing immobilised and control embryos and showed the detailed changes leading to more “flattened” joint surfaces and loss of functional outgrowths; with strongest effects on the lateral femoral condyle where reduced cell proliferation coincides with loss of protrusion (Figure 2). Similar shape analysis in mouse embryos with reduced or absent muscle also showed flattening of characteristic protrusions (Sotiriou et al. 2019), although less pronounced at later stages (Sotiriou et al. 2022). Such changes in shape, also noted in the immobilised zebrafish jaw (Brunt et al. 2016), would impact joint function and stability, potentially further reducing the patterns of normal stimuli generated by movement of the joint and providing an explanation for human developmental disorders associated with reduced foetal movement such as developmental dysplasia of the hip (Giorgi et al. 2015).

As previously noted, Temporary Brittle Bone Disease suffered by human infants with reduced foetal movement can regress during the first year of life as bones strengthen due to postnatal movement (Miller and Hangartner 1999). This suggests a capacity for the system to recover, at least in some respects, if movement resumes, and opens the possibility of physical therapy treatments for such developmental problems (Niles et al. 2019). To investigate the capacity to recover we compared the effects of prolonged immobilisation to a period of immobilisation followed by normal recovery or followed by hyperactivity stimulation in chick embryos and showed that hyperactivity following a period of immobilisation led to reduced joint fusion and commencement of cavitation, particularly at the hip joint (Rolfe et al. 2021). Similarly, external manipulation of immobilised limbs led to improved hip joint morphogenesis and cavitation compared to unmanipulated limbs (Bridglal et al. 2021). The plasticity of the developing joint, contributed to by the response to movement, is also shown by mouse mutants and embryo manipulations where skeletal elements are missing, leading to joints forming within a completely different morphological context e.g. mutant mice that lack Hox11 paralogous genes are missing zeugopod elements (Koyama et al. 2010) and amphibian limb amputations (Tsutsumi et al. 2015). The "new" joints have altered morphology to produce functional interfaces between very different interfacing skeletal rudiments. These studies show the remarkable plasticity of the skeleton in response to its biophysical context and the mechanical loads it experiences.

2.2 Ossification

Embryo movement is required for normal initiation and progression of ossification and mineral deposition, demonstrated in animal models (Hosseini and Hogg 1991b; Nowlan et al. 2008a; Nowlan et al. 2010a) and reflected in the brittle bones of human infants resulting from reduced uterine mobility, leading in some cases to fracture of bones at birth (Rodríguez et al. 1988a; Rodríguez and Palacios 1991; Rodríguez et al. 1988b; Hall 2009) (Figure 2). Nowlan et al showed reduced and abnormally shaped ossification fronts at E14.5 in some skeletal rudiments (Nowlan et al. 2010a). Gomez et al (Gomez et al. 2007) used micro-CT scanning to focus on mineralisation and showed shorter mineralisation zones and less mineralisation in the *Myf5*$^{-/-}$*; MyoD*$^{-/-}$ muscle-less mouse embryo at E18.5. This has consequences for both bone morphology and bone strength. Increased ossification has been shown in cultured femurs under hydrostatic pressure (Henstock et al. 2013). Optimal bone mineral density is controlled through life by a balance between resorption by osteoclasts and deposition by osteoblasts which is known to respond to mechanical input (Frost 2001).

Mineral deposition at the periosteum increases the width and influences the circumferential outline of the bone. Osteoblasts deposit mineral at the periosteal (outer) surface while osteoclasts resorb bone on the inner endosteal aspect. Different rates of mineral deposition in different positions leads to specific cross-sectional shapes and to bone curvature. Several studies have shown simpler, more cylindrical shapes and straighter bones in immobilised embryos (Gomez et al. 2007; Rodríguez et al. 1992; Sharir et al. 2011; Pai 1965b; Hall and Herring 1990; Rot-Nikcevic et al. 2006). Sharir et al (Sharir et al. 2011) monitored structural and mineral changes over normal development in the appendicular bones and showed asymmetric mineral deposition and transient cortical thickening leading to anisotropic circumferential growth and bone curvature. They incorporated these observations into a model for optimal load bearing bone capacity and showed that in *mdg* mouse embryos, devoid of muscle load, the typical circumferential outline of each bone is lost and the bones are mechanically inferior.

So mechanical input from movement builds stronger bones that are optimally shaped (circumferential and curvature) by modulating patterns of mineral deposition.

2.3 Size and shape of skeletal elements

There is an enormous variety of shapes/morphologies among the more than 200 bones of the vertebrate skeleton; each is sculpted as it develops through patterns of localised growth, curvature, ossification, the formation of protrusions (tuberosities) for tendon and ligament attachment and the placement and morphogenesis of joints. Limb long bones in particular derive their individual and complex shapes, with tuberosities for muscle attachment, the need to accommodate articulation at joints and the formation of sesamoid bones that can redirect force and alter movement. The skeletal rudiments are not passive in this sculpting process as their emerging shape and form influences the magnitude and patterns of stimuli generated by muscle movement (Nowlan et al. 2008a). Immobilisation studies have shown that mechanical stimulation from movement influences multiple aspects of shape generation (Figure 2). Impacts on joint formation, ossification, circumferential growth and curvature have already been covered in the previous sections so here the focus will be on longitudinal growth, the formation of tuberosities (entheses) for tendon attachment and sesamoid formation.

Immobilisation in both chick and mouse models results in shorter skeletal rudiments with limb long bones and the scapula affected most (Drachman and Sokoloff 1966; Hall and Herring 1990; Nowlan et al. 2008b; Nowlan et al. 2010a; Pai 1965b; Pollard et al. 2017; Hosseini and Hogg 1991b, a; Rot-Nikcevic et al. 2006; Gomez et al. 2007). Differential effects on individual growth plates lead to changes in limb proportions (Pollard et al. 2017; Hall and Herring 1990) and is responsible for the abnormal protrusion of the lower beak seen in immobilised chick embryos since the upper jaw is reduced more than the lower (Hosseini and Hogg 1991a). The increase in rudiment length when eggs are incubated at higher temperatures are believed to be due to increased embryo motility (Hammond et al. 2007). Longitudinal growth is contributed to by chondrocyte proliferation in the proliferative zone, by the organisation of chondrocytes into elongated columns, by chondrocyte hypertrophy and extracellular matrix (ECM) deposition (Wilsman et al. 1996). Movement has been shown to affect proliferation, chondrocyte column elongation and matrix deposition. The size of the proliferative zone and chondrocyte proliferation are reduced in immobilised chick skeletal rudiments (Roddy et al. 2011a; Germiller and Goldstein 1997). Finite Element modelling based on realistic morphologies captured from the embryo showed that regions of elevated proliferation rates in the rudiment epiphyses corresponds to regions under expansion and regions of high dynamic stimuli generated by muscle contractions (Roddy et al. 2011a). Chondrocyte proliferation and differentiation are controlled by a negative feedback loop between IHH and PTHrP signalling (Vortkamp et al. 1996) and expression of both *Ihh* and *Pthrp* genes were shown to be altered under immobilisation, *Pthrp* upregulated in chick femora (Roddy et al. 2011b) and *Ihh* downregulated in sutured mouse jaws (Jahan et al. 2014). Mechanical influence on chondrocyte proliferation has also been demonstrated in culture (Wu and Chen 2000).

The organisation of chondrocytes into columns whereby daughter cells resulting from cell division intercalate and stack, makes an important contribution to longitudinal growth. Shwartz et al (Shwartz et al. 2012) showed that chondrocyte stacking in zebrafish pharyngeal cartilage, in multiple

immobilisation models, fails to occur, leading to abnormal skeletal morphology. Interestingly in muscle-less limb mouse embryos (*Sp*^{d}) the effect is not as severe since intercalation of chondrocytes and column formation process does occur, but results in shorter columns than normal.

Entheses are sites of ligament and tendon attachment and are prominent shape features of long bones. Tendon attachment sites in particular provide the structurally important interface between tendon and bone, vital for tolerating the high forces of muscle contraction. This is achieved by a gradation of structures from tendon to fibrocartilage, to mineralised fibrocartilage, to bone (Schwartz et al. 2012; Felsenthal and Zelzer 2017). Entheses formation is a good example of the interdependence between muscle tendon and bone involving different types of cross tissue regulation. Entheses are reduced in immobilised mice (Pai 1965b; Nowlan et al. 2010a; Rot-Nikcevic et al. 2006; Gomez et al. 2007; Blitz et al. 2009); while initiation is movement independent, immobilisation leads to arrest of chondrocyte proliferation and structure loss (Blitz et al. 2009). Reduced loading also leads to reduced mineral deposition and fibrocartilage (Thomopoulos et al. 2007; Tatara et al. 2014). The normal graded structure of entheses gives the required mechanical properties, preventing tearing and allowing the transmission of force and, as such, entheses serve as adaptors for the important mechanical interdependency of the tissues (reviewed in (Felsenthal and Zelzer 2017).

Chick immobilisation experiments showed absence of characteristic sesamoids of the hind limb including the knee patella (Drachman and Sokoloff 1966; Hosseini and Hogg 1991a; Roddy et al. 2011b). Sesamoids are small bones positioned within tendons, providing leverage and facilitating distribution of forces and, due to their mechnosensitivity, were believed to form from tendon tissue in response to load. Eyal et al (Eyal et al. 2015) however showed that the patella forms initially as part of the femoral cartilage template and subsequently becomes separated from the femur by a mechanosensitive joint formation process that fails to occur in muscle-less mouse embryos. So this feature of skeletal morphogenesis is due to mechanically sensitivity joint formation.

2.4 Tendon maturation

Tendons are composed of fibrous connective tissue and connect muscle to bone, playing a key role in the integration of the musculoskeletal system and the transmission of loads due to muscle contraction. Tendon homeostasis and repair are known to require appropriate mechanical stimulation with TGFβ signalling playing a key role in the response, activating expression of Scleraxis (*Scx*) transcription factor and collagen target genes (Maeda et al. 2011). During limb development there are multiple examples of cross-regulation between muscle, tendon and cartilage rudiments ensuring the construction of a functional system (Edom-Vovard and Duprez 2004; Huang et al. 2015; Huang et al. 2013; Wang et al. 2010). While the earliest step in tendon development (specification) is independent of muscle contraction, later stages are affected by lack of muscle or immobilisation. Kardon (Kardon 1998) examined muscle and tendon development in chick limbs developing in the absence of either tissue and showed that tendon is required for correct muscle patterning and that in the absence of muscle, proximal and distal tendons are affected differently; while proximal tendons degenerate, distal tendons fail to mature. Immobilisation of embryos consistently shows reduced size and altered structure of tendons (Mikic et al. 2000; Beckham et al. 1977; Germiller et al. 1998; Peterson et al. 2021; Edom-Vovard et al. 2002; Pan et al. 2018) (Figure 2). Havis et al showed that TGFβ signalling

and FGF signalling independently upregulate the tendon marker gene *Scx* and that both are regulated by muscle movement (Havis et al. 2016). Furthermore, the zinc finger transcription factor *Egr1* has been shown to be mechanosensitive (Gaut et al. 2016) and is postulated to lie upstream of the TGF β mechanoresponse.

During development tendon precursor cells (tenocytes) align in the position of the future tendon, forming channels for collagen deposition (Richardson et al. 2007; Kalson et al. 2015). The ratio of cell to ECM reduces over time and at a critical point in later development there is a rapid increase in collagen fibril length coincident with a much stiffer and stronger tendon structure (McBride 1988; Birk et al. 1995; Peterson et al. 2021). This transition is sensitive to embryo movement in the chick where immobilisations result in reduced mechanical modulus and failure to undergo normal developmental maturation (Pan et al. 2018; Peterson et al. 2021). The transition appears to take place later in mouse and is similarly dependent on mechanical stimulation (Theodossiou et al. 2021). This suggests that there is a major reorganisation of the collagenous network during late-stage development that is dependent on movement. We recently showed that this maturation in mechanical properties in chick coincides with a step change in the size of the tendons and the spacing between tendons in the chick hindlimb ankle, as well as changes in the alignment of fibrils, and that in immobilised embryos structural maturation does not take place (Figure 3) (Peterson et al. 2021). Currently our understanding of the molecular and cellular mechanisms involved in transducing the mechanical stimulation from movement that bring about structural and mechanical maturation of the tendon is lacking. At the same time tendon injuries and tendinopathies present major challenges for clinical treatment since repair is frequently limited or not possible (Schweitzer et al. 2018). Tissue engineering attempts have failed to produce a robust load bearing tissue, presumably because of our limited understanding of the regulatory mechanisms that need to be triggered (Bianchi et al. 2021). Furthering our knowledge of the mechanisms involved in tendon construction and maturation in the embryo/neonate holds the promise of recapitulating developmental steps to better effect (Glass et al. 2014).

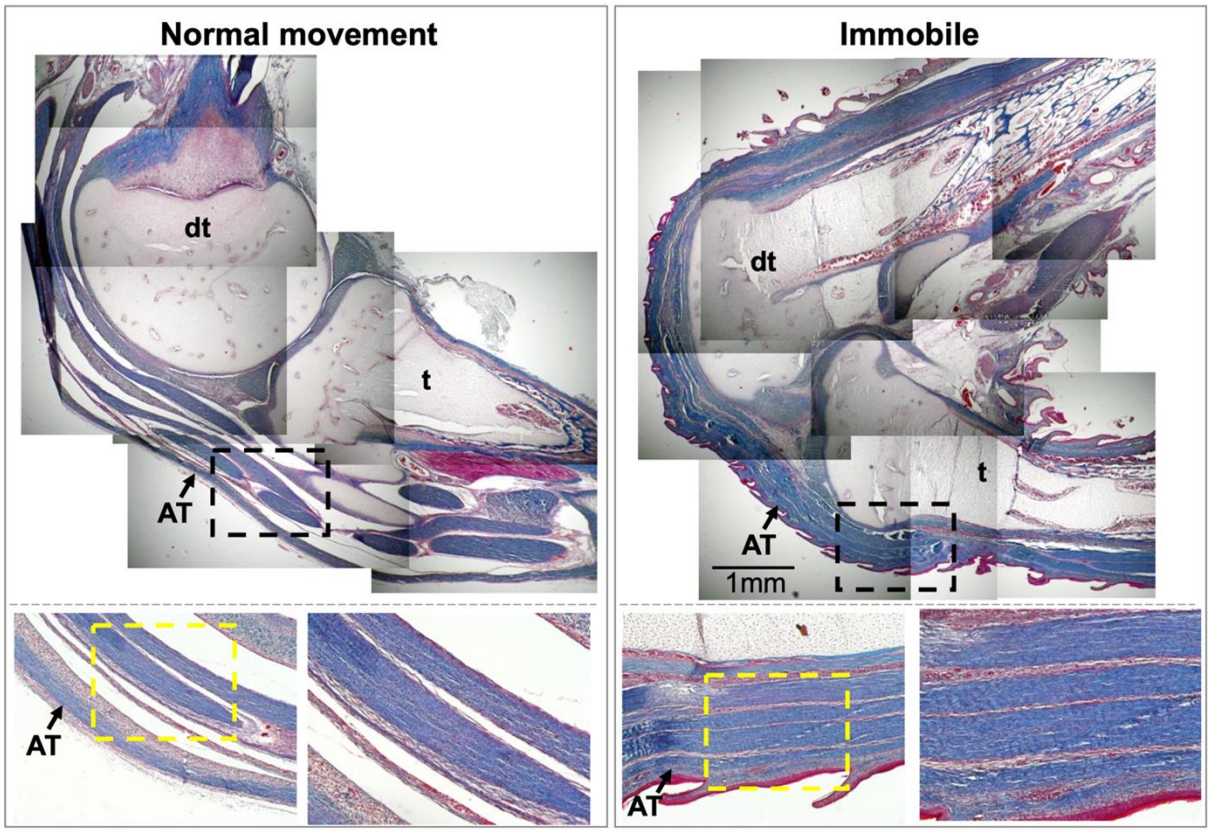

Figure 3: Tendons in the ankle region of control and immobilised embryos at E17 show reduced size and dramatic loss of spacing between tendons. The immobilisation regime used here was daily treatment with decamethonium bromide from E14 to E16. The most dorsal tendon is the Achilles tendon. dt: distal tibiotarsus, t: tarsometatarsus, AT: Achilles Tendon. Scale bar as indicated

3. SECONDARY CARTILAGE FORMATION: SCULPTING FUNCTIONAL SHAPE

In the embryo, bone can develop directly from condensation of mesenchymal cells (intramembranous ossification (or membrane bone)) or by replacing a pre-existing cartilage template (endochondral ossification). The long bones of the limbs that we have been considering in detail form by endochondral ossification. The cartilage template that precedes ossification is referred to as primary cartilage; secondary cartilage arises from the periosteum (or adjacent mesenchyme) and provides a means of growth and morphogenesis of membrane bones, adding functional features (Hinton 2014). In mammals, membrane bones form almost entirely in the craniofacial skeleton, the clavicle being the only membrane bone to form outside. It has been proposed that this predominance of endochondral ossification in the post-cranial skeleton may have evolved to better accommodate fast growth during embryogenesis since membrane bone can only grow from the surface (reviewed in (Hall 1987)). Membrane bones of the facial skeleton, in particular the lower jaw, have been studied extensively.

Another important difference to note about membrane bones of the craniofacial region is that most of them are formed from migrating neural crest cells, in particular all of the facial skeleton, derived within the maxillary, mandibular, and hyoid arches. So, unlike the situation in the limb where the migratory cells give rise to muscle and the endogenous cells form the skeleton, in the facial region it is the opposite- the migratory neural crest form the skeleton while the muscle forms from resident

cells. Also note that the appearance of secondary cartilage derived from the periosteum of membranous bone involves a switch from osteogenesis to chondrogenesis.

Secondary cartilage develops on craniofacial membrane bones, including the mandibles, and also on the mammalian clavicle. Mandibles and clavicles are the first bones to ossify (Hall 1987) and demonstrate secondary cartilage formation that is particularly sensitive to mechanical stimulation from movement. The mouse mandible has three sites of secondary cartilage formation; the condylar, coronoid and angular processes of the lower jaw, while the clavicle has a single site. Chick embryo immobilisation showed that the clavicle is among the most strongly affected bones analysed, both in terms of growth reduction and absence of secondary cartilage initiation (Hall 1986; Hall and Herring 1990). In *mdg* mice both clavicles and mandibles are reduced and abnormally shaped, with indication that sites of secondary cartilage initiate but do not maintain (Pai 1965a; Herring and Lakars 1982). Rot-Nikcevic et al carried out a detailed analysis of clavicles and mandibles in mouse embryos developing in the absence of muscle, recording alterations in the morphology, and asking if initiation and/or maintenance of secondary ossification sites are affected (Rot-Nikcevic et al. 2007). They showed that the effects differed according to rudiment and site going from absence of initiation of the angular process to dramatically reduced size of the condylar and coronoid processes (Figure 2). A microarray comparison between transcripts in amyogenic and wildtype mandibles identified candidate genes involved in muscle dependent mandibular development (Rot et al. 2014). Sutured mouse embryonic jaws also showed reduced and deformed condylar processes (Jahan et al. 2014).

The sites and details of secondary cartilage differ between mammals and birds. In birds, secondary cartilage sites are common where membrane bones form articulations and there is a great variety in their precise shape and placement across birds with different feeding habits. This diversity can offer insights into the importance of mechanically directed developmental plasticity in sculpting functional morphologies (Solem et al. 2011). Ducks, who feed by levered straining, have a prominent bony process on the lateral surface of the lower jaw, at the point of muscle attachment. On the other hand, chickens and quails who feed by pecking have only a small bony ridge. Solem et al showed that paralysis of duck embryos led to loss of the characteristic process resulting in a smoother mandible resembling that of the chick (Solem et al. 2011). A study of cichlid fish examined associations between facial bones and muscles using 3-dimensional morphometrics (Conith et al. 2019). They found that the shape of some bones appears to be more sensitive to muscle load than others. They also show strong associations that go beyond the points of muscle insertion implying that muscle load influences broader features of the craniofacial skeleton and that changes in the biophysical environment during development or through the life of the animal could alter morphology. Such changes could be due to heritable changes affecting development (e.g changing features of adjacent tissues) or due to changes in a novel environment. It is interesting in this context that zebrafish jaw shape was shown to be particularly sensitive to movement (Shwartz et al. 2012; Brunt et al. 2016). Jaw shape in mice has also been shown to be altered in response to mastication forces stimulating osteocyte differentiation (Inoue et al. 2019).

The interdependence of muscle and skeleton in sculpting facial structures is also illustrated by neural crest cell interspecies transplantation experiments where quail neural crest are transplanted into a duck embryo leading to quail derived facial skeleton with quail shape, but also muscle pattern and attachment sites secondarily altered to the quail pattern (Tokita and Schneider 2009; Solem et al. 2011).

4. EFFECTS OF REDUCED MOVEMENT ON THE SPINE

Less is known about the influence of foetal movements on the spine although spinal deformities have been described in conditions and syndromes of reduced *in utero* foetal movement such as FADS/ Pena Shokeir syndrome (Bisceglia et al. 1987; Gupta et al. 2011; Hall 2009; Ochi et al. 2001; Persutte et al. 1988; Tomai et al. 2017) and arthrogryposis (Hall 2014; Ma and Yu 2017; Nouraei et al. 2017; Yfantis et al. 2002). Spinal defects associated with foetal akinesia include curvature abnormalities (Ma and Yu 2017; Zhang et al. 2021; Hall 2014; Tomai et al. 2017; Persutte et al. 1988; Ochi et al. 2001; Nouraei et al. 2017) and failed development of vertebrae (Bisceglia et al. 1987; Crane and Heise 1981). The curvature abnormalities observed due to foetal akinesia include scoliosis (abnormal lateral curvature) (Nouraei et al. 2017; Yfantis et al. 2002; Zhang et al. 2021) and lordosis (abnormal ventral curvature) (Nouraei et al. 2017; Tomai et al. 2017) and range phenotypically from mild to severe, with suggestions that the observed variability in effects depends on the developmental window in which movement is interrupted (Filges et al. 2019). Kyphosis (abnormal dorsal curvature) is not associated.

As with effects on forming joints, ossification and skeletal shape, embryonic immobilisation models also show abnormalities of the developing spine that reflect clinical observations. Spinal curvature abnormalities were described in early chick immobilisation studies (Murray and Drachman 1969; Sullivan 1974, 1966; Hosseini and Hogg 1991a) including 'the lateral bending of the cervical spine, rotated about its own axis'(Murray and Drachman 1969) and 'the buckling of the vertebral column into an S-shaped curvature'(Sullivan 1966). The structural and functional descriptions of paralysis associated with these abnormalities include: immobile neck-joints (Drachman and Coulombre 1962), absent articulations of the vertebral bodies and ill-defined articulation between the vertebral articular processes in the cervical spine (Murray and Drachman 1969), fusions and asymmetries of cervical vertebrae (Sullivan 1974; Hosseini and Hogg 1991a), extensive union between vertebrae, and absent articulation of the first cervical vertebra (Sullivan 1966) (Figure 2). Similarly, altered foetal movement in mammalian embryonic environments further support *in utero* movement playing a critical role in spinal morphogenesis (Moessinger 1983; Panter et al. 1990). Induction of foetal akinesia in a rat model reported contractures in the spine and tail, with the neck in a permanent flexed position (Moessinger 1983). Spinal contractures including torticollis (wryneck), scoliosis and lordosis, especially in the cervical and lumbar regions due to the wedging of vertebrae were observed in an immobile embryonic goat model induced through the maternal consumption of conium seed and nicotiana glauca (Panter et al. 1990); similarly reported in calf and porcine development (Keeler 1974; Panter et al. 1985). The duration of reductions in foetal movements are an important factor in the severity and permeance of deformities observed, particularly in the spinal column, with short term foetal immobility resulting in modest to moderate phenotypes that resolved by 8-10 weeks post-partum (Panter et al. 1990).

The murine embryonic models with absent or immobile muscle also display characteristic spine malformations of reduced foetal movement. Postural anomalies including enlarged and fused cervical vertebrae are present in embryos that have a complete absence of striated muscle (*Myf5*$^{-/-}$*:MyoD*$^{-/-}$*)*(Rot-Nikcevic et al. 2006), while immobile muscle in *mdg* embryos show defects in cervical, thoracic and lumbar vertebral segmentation including vertebral body fusion (Herring and Lakars 1982; Kahn et al. 2009; Pai 1965b) and abnormal development of intervertebral discs (Levillain et al. 2021).

Alterations in the alignment of collagen fibres and mechanical properties of the annulus fibrosus are also observed in the cervical region of immobile *mdg* embryos at later stages (Levillain et al. 2021).

It has been over 50 years since spinal defects were listed among the skeletal malformations resulting from chick embryo immobilisations but only recently have more detailed studies been performed, focusing on the specific effects of muscle forces on the spine (Rolfe et al. 2017; Levillain et al. 2019; Rolfe et al. 2021). Quantification of spinal curvature defects using three-dimensional imaging of paralysed spines, as an analogy for Cobb angle measurements performed in abnormal human curvatures, identified significant kyphotic and lordotic curves following paralysis (Rolfe et al. 2017; Levillain et al. 2019; Lonstein 1999). Defects were observed in the cervical, thoracic and lumbar regions, the cervical region being the most severely affected (Levillain et al. 2019; Rolfe et al. 2017; Rolfe et al. 2021). The onset and duration of paralysis impacted the severity of defects in the spine, including the extent of curvature abnormalities, number and site of vertebral fusions, extent of vertebral wedging and vertebral shape changes (Figure 2)(Levillain et al. 2019; Rolfe et al. 2017). The most severe defects were observed with paralysis at early stages of spine patterning (Levillain et al. 2019; Rolfe et al. 2017; Rolfe et al. 2021), with later and shorter periods of paralysis resulting in milder to moderate phenotypes, further supporting that the timing of foetal motility is a critical influence on the normal development of the spine, aligning with the variability seen clinically following foetal akinesia (Filges et al. 2019; Rolfe et al. 2017). The ability of the spine to recover if movement resumes was investigated through induced movement following immobility, but did not result in any spinal improvements under the conditions investigated (Rolfe et al. 2021). However, given the variability in defects observed, dependent on the timing of initiation and duration of paralysis, the capacity to recover following resumption of movement at different time points needs to be more fully assessed.

Combining clinical and experimental evidence, it is clear that foetal movement plays an important role in normal morphogenesis of the spine. Interruptions in foetal movement, at critical windows, leads to congenital spine defects. Much remains to be investigated, including the molecular mechanisms impacted by alterations in the mechanical environment in the spine. Another important consideration is the impact on supporting connective tissues, essential for physiological function, such as the stabilising vertebral ligaments. Investigation of the mechanisms involved could further our understating of the origins of idiopathic scoliosis, where it is possible that mild or undiagnosed congenital spinal deformities might contribute to the progressive nature of conditions presenting in adolesence.

5. THE MECHANISMS THAT TRANSDUCE THE EFFECTS OF MUSCLE MOVEMENT IN THE DEVELOPING SKELETON

Mechanical loading can produce a variety of biophysical stimuli- such as compression, tension, hydrostatic pressure, osmotic pressure, and fluid shear stress. How these stimuli are sensed by the cell and transduced to a biological output has been challenging to address. As reviewed in previous sections, a number of candidate regulatory pathways within the responding cells have been shown to be fundamentally altered, in particular the precise localisation of BMP and Wnt signalling at the joint which is lost under multiple immobilisation scenarios across chick and mouse (Singh et al. 2018; Rolfe et al. 2018). This shows how cells at the joint line are misdirected by inappropriate mechanical cues, resulting in joint fusions.

A transcriptomic study was designed to search for genes that are differentially regulated in the immobile humerus and associated joints of muscle-less mouse embryos, compared to wildtype littermates(Rolfe et al. 2014). As previously mentioned Wnt and BMP pathway genes were strongly impacted. Genes associated with the cytoskeletal architecture and with Hippo/YAP signalling were also altered. Genes encoding integrins, cadherins and other ECM associated proteins were down regulated in immobilised tissue (Rolfe et al 2014).

YAP signalling is also of particular interest as a candidate mechanism because of its association with sensing the biophysical environment in other biological contexts (Nishioka et al. 2009; Dupont et al. 2011; Halder et al. 2012). Looking closer at YAP signalling we showed differential localisation of active YAP and YAP target gene expression associated with regions of growth in the enlarging condyles, which is altered in the absence of movement (Shea et al. 2020). This could be a contributory mechanism to altered shape of rudiments at joint interfaces.

Less is known about how mechanical cues alter these effector pathways; what mechanism(s) mediates the cues at the cell surface. Candidate mechanosensory mechanisms include integrin signalling (cell-ECM interactions), cadherin signalling (cell-cell interactions), and mechanosensitive ion channels that respond to physical changes in the cell membrane (stress activated) (reviewed in (Dieterle et al. 2021). A number of different stress activated ion channels could together cover a broad spectrum of stresses in skeletal cells: Piezo1 and Piezo2 respond to high levels of strain and cell deformation while TPRV4 can transduce more physiological levels of strain (Kanju et al. 2016; Lee et al. 2014). It is interesting that mutation in Piezo2 leads to arthrogryposis and Walker syndrome characterised by kyphoscioliosis and joint contractures (Chesler et al. 2016; Ma et al. 2023). Recently, involvement of the TRPV4 Ca2+ ion channel in sensing movement has been demonstrated in mouse limb explants in culture (Khatib et al. 2023). This study showed that TRPV4 is expressed in the developing rudiment where its accumulation is sensitive to movement and that addition of a TRPV4 antagonist could reduce the shape features that are responsive to movement.

Another mechanism that could sense physical stimuli is the primary cilium which projects outward from the cell surface and senses the mechanical environment (reviewed in (Spasic and Jacobs 2017)). In multiple contexts, primary cilia act as foci for Hedgehog (Hh), Wnt, and calcium signalling, with pathway components localised to the cilium (reviewed in (Bangs and Anderson 2017; Elliott and Brugmann 2019)). The physical characteristics of the cilium indicate a role in mechanosensing with the axoneme projecting into the extra-cellular space where It can interact with components of the extra cellular matrix as well as ligands or other diffusible molecules. During development, the primary cilium senses fluid flow at the embryonic node, playing a role in left-right asymmetry, as well as calcium signalling during kidney development (reviewed in (Bisgrove and Yost 2006). In growth plates of chickens, ciliogenesis is Induced by loading (Rais et al. 2015). But less is known about the primary cilium on the developing skeletal rudiments. Although no functional relationship has been shown to date, we have demonstrated localised patterns of primary cilia on the chondrocytes of the developing mouse skeletal rudiments at stages when development is impacted in muscle-less mutants (Shea and Murphy 2021).

6. IMPLICATIONS FOR FUTURE WORK

The final shape and size of each bone is sculpted by mechanical cues from the musculature. During development of the musculoskeletal system there is close co-ordination and interdependence between the emerging tissues with reciprocal cross regulation between the forming musculature and skeleton. Focus here has been on the environmental effects of mechanical stimulation from movement, i.e., the influence of contracting muscles on tissue patterning, shape, structure and mechanical properties of the component elements of the skeleton, with examples from the developing limb, jaw and spine. The remarkable plasticity of the skeletal system in response to mechanical load from the musculature that this cumulative body of work reveals, is important for building an integrated system that ensures structural and functional compatibility between the elements and allows co-ordinated evolution.

Developmental plasticity builds a finely tuned functional system, ensuring the capacity to respond to changes and fluctuations (West-Eberhard 2005a, 2003). The skeletal system has such remarkable plasticity that behavioural changes, perhaps in response to a changed environment, could bring about morphological change. West-Eberhard argues that morphological changes can take place in response to environmental change acting within the plasticity of the evolved system, and that such changes could then be subject to selection with the addition of further heritable change. She cites the report of Dutch anatomist Slijper of a goat with malformations of the upper limbs that adopted bipedal movement and upon autopsy showed extensive co-ordinated changes throughout the musculoskeletal system (West-Eberhard 2005b, 2003).

The focus of Neo-Darwinism on gradual adaptation of existing traits falls short in providing an explanation for the origin of novelty; developmental plasticity and skeletal response to mechanical cues can provide such an explanation where a new structure would not require independent genetic mutations affecting each of the component tissues: rather a change affecting one tissue, its structure or performance, could impact other elements, maintaining a functional, integrated system. For example the ectopic formation of cartilage or bone within tissues that have chondrogenic capacity could give rise to extra skeletal elements such as sesamoids and in the case of the giant panda, the radial sesamoid in the wrist was co-opted to form an extra digit (Wang et al. 2022). The morphological innovation giving the “apposable” digit 1 in birds, allowing the grasp required for perching, is achieved by twisting of the metatarsal during development and this is lost following paralysis of chick embryos (Francisco Botelho et al. 2015). Another example is the loss of muscles and associated joints that occurred during the evolution of cetacean fins (Cooper et al. 2007).

Waddington coined the term “epigenetic landscape” to represent the network of regulatory interactions involved in cell fate and tissue patterning decisions (reviewed in (Siegal and Bergman 2002; Tronick and Hunter 2016). Such networks of gene interaction, including input from environmental stimuli, connect the genotype to the phenotype and, importantly, it is the phenotype that can provide a selective advantage. Skeletal developmental plasticity is a great example to explain and further explore such concepts (Davila-Velderrain et al. 2015).

Congenital abnormalities resulting from reduced foetal movement are heterogeneous and present several difficulties in terms of prenatal diagnosis and management (Niles et al. 2019). The demonstrated plasticity of skeletal development and the capacity for recovery indicated in experimental models gives hope for possible remedial intervention, especially in the case of extrinsic causes and milder abnormalities. More detailed information on the capacity for recovery and the

threshold levels of stimulation needed for normal development would be very valuable. The large number of aspects of skeletal development that can be impacted by reduced movement including, joint function, bone strength and spinal curvature makes intervention more complicated but the progressive nature of conditions such as joint dysplasia and scoliosis highlights the importance of early diagnosis and remedial intervention.

Tissue engineering approaches to regenerate skeletal tissues including cartilage, bone and tendon, hold great promise in the treatment of widespread skeletal conditions caused by damage or disease. Progress has been made on some fronts but major barriers still exist, often related to our limited understanding of what is required to direct stable cell differentiation and functional tissue assembly. The approach not only needs to provide cells with the potential to form specific tissues but also the environment that encourages appropriate differentiation, trophic and mechanical. The appropriate treatment regimen is vital. We need to understand fully the biological properties of the cells and how they respond to all aspects of the environment to be able to recapitulate essential aspects of the system. While our knowledge of the effects of mechanical stimulation on many aspects of skeletal development has increased enormously in recent years and aspects of the molecular mechanisms involved have been uncovered, we need to have a fuller understanding of the key cellular responses involved. This needs to be a major focus for future work to realise the promise to regenerative therapies.

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