

## **Mechanobiology of Embryonic Skeletal Development: Insights from Animal Models**

For Publication in issue on: "CURRENT ANALYSES OF EMBRYONIC DEVELOPMENT"

Niamh C. Nowlan<sup>1,2\*</sup>, James Sharpe<sup>1</sup>, Patrick J. Prendergast<sup>2</sup>, Paula Murphy<sup>2,3</sup>

1. EMBL-CRG Systems Biology Unit, Centre for Genomic Regulation (CRG), Dr. Aiguader, 88, 08003 Barcelona, Spain.
2. Trinity Centre for Bioengineering, School of Engineering, Trinity College Dublin, Ireland.
3. Department of Zoology, School of Natural Sciences, Trinity College Dublin, Ireland.

\*Address for correspondence: Niamh Nowlan, EMBL-CRG Systems Biology Unit, Centre for Genomic Regulation (CRG), Dr. Aiguader, 88, 08003 Barcelona, Spain. Phone: +34-933-160-100; Fax: +34-933-969-983; E-mail: [nowlann@tcd.ie](mailto:nowlann@tcd.ie)

**Abstract (limit 250 words)**

A range of clinical conditions in which foetal movement is reduced or prevented can have a severe effect on skeletal development. Animal models have been instrumental to our understanding of the interplay between mechanical forces and skeletal development, in particular the mouse and the chick model systems. In the chick, the most commonly used means of altering the mechanical environment is by pharmaceutical agents which induce paralysis, while genetically modified mice with non-functional or absent skeletal muscle offer a valuable tool for examining the interplay between muscle forces and skeletogenesis in mammals. This article reviews the body of research on animal models of bone or joint formation *in vivo* in the presence of an altered or abnormal mechanical environment. In both immobilised chicks and ‘muscleless limb’ mice, a range of effects are seen, such as shorter rudiments with less bone formation, changes in rudiment and joint shape and abnormal joint cavitation. However, while all bones and synovial joints are affected in immobilised chicks, some rudiments and joints are unaffected in muscleless mice. We propose that extrinsic mechanical forces from movements of the mother or littermates impact on skeletogenesis in mammals, while the chick embryo is reliant on intrinsic movement for mechanical stimulation. The insights gained from animal models into the mechanobiology of embryonic skeletal development could provide valuable cues to prospective tissue engineers of cartilage and bone, and contribute to new or improved treatments to minimise the impact on skeletal development of human disorders of reduced movement *in utero*.

**Key Words:** Mechanical forces; muscle contractions; bone; joints; ossification; immobilization.

## Introduction

The importance of mechanical forces for embryonic development was central to the thinking of developmental biologists around the turn of the 20<sup>th</sup> century. In 1888, Wilhelm His (1831–1904) stated that “*to think that heredity will build organic beings without mechanical means is a piece of unscientific mysticism*” (His, 1888). In 1894, Wilhelm Roux founded a journal called the Archives for Developmental Mechanics (Archiv für Entwicklungsmechanik) and defined the goal of developmental mechanics as “*the ascertainment of formative forces or energies*” (Roux, 1894). With the revolution brought by modern molecular genetics, the idea that mechanical forces are key to development fell quickly out of favour among much of the developmental biology community, being perceived as outdated (Gould, 1985), with an emphasis on cellular events being guided by positional information based on molecular cascades. The last few decades of research have indeed revealed the central role played by complex networks of regulatory molecules which direct the development of finely-tuned, integrated systems in the embryo. However, cells also receive input from the environment of the emerging tissues, including mechanical information in the form of biophysical stimuli such as stress, strain and fluid flow. Although awareness of mechanical influences is gradually increasing, in general, the study of morphogenesis is still dominated by a molecular perspective. Little is known about how mechanical cues are integrated with molecular regulation of gene expression guiding local cellular events such as differentiation, shape change, proliferation and apoptosis during development (Henderson and Carter, 2002), but it is clear that increased knowledge on the mechanobiology of embryogenesis will provide a fuller understanding of developing systems. One aspect of developmental biology for which the importance of mechanics has consistently been demonstrated and acknowledged is skeletogenesis, with studies from as early as the 1930s illustrating the importance of the mechanical forces for skeletogenesis (e.g., Fell and Canti, 1934; Murray and Selby, 1930). Findings from clinical conditions, *in vivo* animal models and *in vitro* studies have confirmed the vital role that mechanical forces play in skeletal development. In recent years, there has been an explosion in interest in the interactions between mechanical forces and cells and tissues, particularly from researchers in the tissue engineering field, who would hope to recapitulate the processes of cartilage and bone developmental *in vitro* (Ingber et al., 2006; Kelly and Jacobs, 2010; McMahon et al., 2008).

A range of syndromes and conditions in which foetal movement is reduced, restricted or prevented have provided clinical evidence for the importance of muscle contractions for normal bone and joint formation. Conditions affecting the neuromuscular system, such as congenital myotonic dystrophy (Wesstrom et al., 1986) or spinal muscle atrophy (Nicole et al., 2002), can have a dramatic effect on skeletal development leading to smaller, thinner and weaker long bones, prone to postnatal fracture (Rodriguez et al., 1988a; Rodriguez et al., 1988b). Children suffering from hemiplegic cerebral palsy, where half of the body is affected by compromised motor functions, exhibit delayed skeletal maturation on the affected side compared to the unaffected side (Roberts et al., 1994). Foetal akinesia deformation sequence (FADS) describes the range of symptoms caused by reduced, restricted or absent movement *in utero*, which include craniofacial and limb abnormalities and multiple joint contractures (Hall, 1986; Hammond and Donnenfeld, 1995). Temporary brittle bone disease in infants, which can cause multiple unexplained fractures, has been attributed to decreased or restricted foetal movement (Miller and Hangartner, 1999). Approximately 10% of preterm infants with very low birth weight suffer from low bone density and increased susceptibility to fractures in the first months of life (Dabezies and Warren, 1997), and it has been proposed that it is the removal of the loads normally induced by kicking against the uterine wall during late development that are responsible for the ‘bone disease of preterm birth’ (Miller, 2003). It has also been shown that increased mechanical loading can improve the skeletal development of preterm infants; Moyer-Mileur et al. (2000) showed that a daily physical activity program in preterm infants, where passive resistance was applied to the extremities for 5–10 minutes per day, resulted in increased forearm bone length, bone area and bone mineral content compared to preterm infants who did not undergo the exercise program. Developmental dysplasia of the hip (DDH) is thought to occur in as many as 1 in 100 newborns (Homer et al., 2000), and its incidence and severity is widely thought to be influenced by the mechanical environment *in utero* (Shefelbine and Carter, 2004). While a family history does increase susceptibility to DDH (Weinstein, 1987), the risk of DDH is also increased if joint abduction is limited due to foetal position *in utero*, oligohydramnios (a deficiency of amniotic fluid), or swaddling after birth in a legs extended position (Vanden Berg-Foels et al., 2006).

Animal models are a vital tool with which to increase our understanding of clinical conditions in which an abnormal mechanical environment affects skeletal development. Studying

the interplay between mechanics and the formation of cartilage, bones and joints during development enhances our understanding of the mechanobiology, or biophysics, of the system, and could provide valuable information to prospective tissue engineers of cartilage and bone. Recent findings using animal models show that mechanical input affects patterning and differentiation events as well as the healthy progression of skeletogenesis indicated by the clinical evidence above. In this paper, we review the state of the art research on the interplay between mechanics and skeletal development, with particular focus on animal models of bone or joint formation *in vivo* in the presence of an altered or abnormal mechanical environment.

### **Animal Model Systems for Investigation of Mechanics of Skeletogenesis**

The mouse and the chick are the most commonly used animal model systems for the investigation of the effect of mechanical forces on skeletal development, and each model provides a different set of advantages and suitable approaches. The chick system provides the advantages of an externally laid egg, where the embryo is readily available and amenable to manipulation such as surgically or drug induced paralysis. The mouse is the most widely used and best established system for studying mammalian development and has the benefits of elegant genetic manipulations and a wealth of molecular tools. Genetically modified mice offer a valuable tool with which to examine skeletogenesis when muscle is altered, reduced, or absent. A range of mouse mutants originally generated to examine the function of particular developmental genes that lead to a muscle phenotype can now be used to address the question of how skeletogenesis proceeds in an altered mechanical environment, as listed in Table 1. In *Pax3<sup>Sp/Sp</sup>* (*Spotch*), absence of the transcription factor *Pax3* means that migration of the muscle progenitor cells into the limb buds does not occur and no skeletal muscle develops (Franz et al., 1993; Tajbakhsh et al., 1997). In double knockouts of *Myf5* and *MyoD* (*Myf5<sup>nlacZ/nlacZ</sup>:MyoD<sup>-/-</sup>*) muscle progenitor cells migrate but do not differentiate into myoblasts and therefore muscle fibers are absent (Kablar et al., 2003; Kassam-Duchossoy et al., 2004). In littermates of the double knockouts with one functional copy of *Myf5*, (*Myf5<sup>nlacZ/+</sup>:MyoD<sup>-/-</sup>*), skeletal muscle is present but with a reduction in muscle fibre number of between 35–55% (Rudnicki et al., 1993). Muscular dysgenesis (*mdg/mdg*) is a mutation of the dihydropyridine receptor (DHPR)  $\alpha 1$  subunit which causes a lack of excitation-contraction coupling and therefore muscle contractions are absent in *mdg* mutant mice (Pai, 1965a; Powell et al., 1996). Dock 1 mutants have defective

muscle organisation so that skeletal muscle cells differentiate but do not fuse into mature contracting fibers (Laurin et al., 2008).

The chick embryo has been used extensively as a model system for examining skeletal development in the presence of an altered mechanical environment due to the ease with which the growing embryo can be accessed. The most commonly used treatments are listed in Table 1. Neuromuscular blocking agents can be used to prevent or increase muscle contractions in the developing chick. Rigid paralysis, where muscles are in continuous tension, is most commonly induced by administration of decamethonium bromide/iodide (e.g., Germiller and Goldstein, 1997; Hall and Herring, 1990; Hosseini and Hogg, 1991a; b; Mikic et al., 2000a; Nowlan et al., 2008b), but other agents such as botulinum toxin (Drachman and Sokóloff, 1966; Murray and Drachman, 1969), succinylcholine (Ruano-Gil et al., 1978) and tubocurarine (Hall, 1972) have also been used. Flaccid paralysis, where muscles are relaxed, can be induced using pancuronium bromide (Osborne et al., 2002). Reserpine, an antihypertensive drug which induces hypermotility at low doses and paralysation at high doses, has also been used for *in ovo* experiments (Ruano-Gil et al., 1985). The accessibility of the chick means that surgical techniques can be used to induced immobilization, for example by excision of portions of the neural tube involved in enervation of the limb muscles (Wong et al., 1993) or extirpation of the lumbosacral spinal cord (Drachman and Sokóloff, 1966), which induces immobilization of the lower limbs. However, while working with the chick embryo system has many advantages, it is not ideal for examining endochondral ossification due to the fact that in the growth plates, post-hypertrophic regions are not replaced by bone but are instead resorbed to form a hollow bone (Fell, 1925; Nowlan et al., 2007). Furthermore, long bone secondary centres of ossification are uncommon in the chick (Blumer et al., 2005; Doménech-Ratto et al., 1999; Hogg, 1980). Although surgical interventions are challenging in mammalian embryos, the effects of immobilization in the rat embryo have been studied using subcutaneous injection *in utero* of a neuromuscular blocking drug (tubocurarine) (Rodriguez et al., 1992) and also by induction of oligohydramnios via daily removal of amniotic fluid (Palacios et al., 1992).

## **Bone Development**

Two types of bone develop in the embryo; endochondral and intramembranous bone. Intramembranous ossification occurs when bone forms directly from mesenchymal cells, and this

mode is responsible for most of the craniofacial bones (Jee, 1988). Endochondral ossification occurs when a cartilage template is replaced by bone in a precise series of events, in which chondrocytes progress from proliferating to hypertrophic states, and the resulting hypertrophic cartilage is then mineralised and replaced by bone (Hall, 1987). While the long and short bones of mammals are formed primarily by endochondral ossification, the periosteal bone collar forms via a type of intramembranous ossification (Ham and Cormack, 1979). In birds, ossification of the long bones occurs via intramembranous ossification of the periosteum, where the cartilage in at the core undergoes the same steps of endochondral ossification prior to the mineralisation of hypertrophic chondrocytes, but the matrix of post-hypertrophic chondrocytes is resorbed instead of being mineralised (Fell, 1925; Hall, 1987; Murray and Selby, 1930). Despite the differences in bone development between the two model systems, we and others have shown the effect on bone formation of an altered mechanical environment in both chick and mouse. In particular, decreased rudiment length and reduced bone formation, in both paralysed and muscleless situations in both systems, as detailed below.

Immobilization of chick embryos using neuromuscular blocking agents has been used extensively to examine bone development in the absence of dynamic mechanical stimulation from muscle contractions. The most commonly used agent, decamethonium bromide, induces rigid paralysis, where all muscles are in continuous tetanus (Osborne et al., 2002). In the absence of muscle contractions the muscle mass decreases (Hall and Herring, 1990), and therefore chick embryos immobilised with decamethonium bromide will not only lack dynamic muscle contractions, but will have muscles of lower cross sectional areas and therefore lower muscle forces. A landmark study from Hall and Herring (1990) illustrated the dramatic effect paralysis has on the developing chick skeleton. Immobilization using decamethonium iodide from 7, 8 or 9 days of incubation resulted in abnormal curvature of the mandible, neck and spine. As also reported by Murray and Drachman (1969), the lower beak protruded below the upper beak in immobilised chicks, contrary to the normal situation (Hall and Herring, 1990). The clavicles of the paralysed chicks were thinner and straighter than in controls, ribs were prematurely ossified and fused to the sternum, and the sterna were grossly malformed (Hall and Herring, 1990). Growth rates of the clavicle, mandible, femur, tibia and humerus were decreased in immobilised embryos, and the authors found a differential effect on the rudiments, with the clavicle being the most affected and the mandible the least (Hall and Herring, 1990). Quantification of the effects

of treatment on the muscle masses indicated that the most severe decreases in muscle mass correlated with the more severely affected skeletal rudiments (Hall and Herring, 1990). Similar results were obtained by Hosseini and Hogg (1991a), who found that the lengths of the radius and ulna were also decreased by immobilization. The scapula and pelvic girdle were deformed by paralysation. Ossification in the femur, tibia, humerus, radius and ulna was found to be significantly reduced in immobilised chicks from day 15 onwards, although the timing of appearance of ossification centres was unaffected by treatment (Hosseini and Hogg, 1991a). A detailed study of ossification of the tibia revealed fewer layers of periosteal bone at the mid-diaphysis of the tibia from day 12 in paralysed chicks (Hosseini and Hogg, 1991b). Rates of cartilage and bone formation were significantly reduced in immobilised embryos from day 14 of incubation (Hosseini and Hogg, 1991b). A more recent study from our group focussed on early ossification of the chick tibiotarsus and showed reduced bone proportion in immobilised embryos (Nowlan et al., 2008b).

Hindlimb muscular atrophy was induced by excising the lumbrosacral portion of the neural tube after 72 hours of incubation (Wong et al., 1993). The femur and tibiotarsus were significantly shorter in experimental animals than in sham-operated controls, and greater flaring at the distal femur was observed. The mechanical integrity of the tibiotarsus was assessed, and the whole bone stiffness, bending strength at failure and brittleness were found to be lower in the tibiotarsi of immobilised embryos (Wong et al., 1993). Germiller and Goldstein (1997) investigated the cellular events underlying the changes observed in the tibiotarsus of immobilised chicks. Cell proliferation was found to be reduced in the proliferative and resting chondrocytes regions of immobilised chicks, while cell density was not significantly affected (Germiller and Goldstein, 1997). The thickness of the proliferative zone was also significantly reduced in immobilised animals, and the authors conclude that the proliferation and/or recruitment of immature chondrocytes is mediated through the action of muscle contractions (Germiller and Goldstein, 1997). In our laboratory, we used finite element analysis to characterise the mechanical environment induced by muscle contractions, and found that dynamic patterns of biophysical stimuli co-localise with regions of incipient bone formation in the embryonic tibiotarsus (Nowlan et al., 2008a). We further supported a prediction that these localised biophysical stimuli are involved in regulating ossification by demonstrating reduced tibiotarsal ossification, combined with dramatic decreases in the levels of predicted biophysical stimuli, in



immobilised embryos (Nowlan et al., 2008b). The expression patterns of two genes involved in bone formation, Collagen X (ColX) and Indian hedgehog (Ihh) showed co-localisation with peak regions of biophysical stimuli, and expression patterns of both genes were altered following immobilization, demonstrating the involvement of ColX and Ihh in mechanoregulatory pathways during embryonic bone formation (Nowlan et al., 2008b)

Immobilization of mammalian embryos *in utero* is challenging, but has been performed in rat embryos (Palacios et al., 1992; Rodriguez et al., 1992). These studies, from the same laboratory, describe immobilization of rat embryos from a relatively late stage in skeletogenesis (embryonic day 17) to term. Rodriguez and colleagues (1992) immobilised embryos *in utero* by daily subcutaneous administration of D-tubocurarine. The femora of immobilised embryos were shorter than sham-operated littermate controls, and exhibited a decreased and rounder cross-sectional area, with impeded periosteal and trabecular bone formation (Rodriguez et al., 1992). Palacios et al. (1992) induced oligohydramnios by daily removal of amniotic fluid and examined the effect of restricted movement on bone development in the femur. In contrast to the previous study, no significant differences were found in femoral length or width, or in periosteal or cortical bone formation, compared to sham-operated littermates (Palacios et al., 1992). The authors conclude that the local application of muscle forces to the rudiment is more important to bone development than the limb movement prompted by those muscle contractions (Palacios et al., 1992).

Genetically modified mice with absent or non contractile musculature have provided major insights into the numerous effects of mechanical stimulation from muscle contractions on mammalian bone development (Gomez et al., 2007; Nowlan et al., 2010; Pai, 1965a; Rot-Nikcevic et al., 2007; Rot-Nikcevic et al., 2006). The first comprehensive analysis of skeletal development in mouse mutants with abnormal skeletal muscle were published in 1965 (Pai, 1965a; b) in a spontaneously occurring mutant line. In muscular dysgenesis (*mdg*) mice, skeletal muscle development initiates largely normally, but muscle contractions do not occur and muscle bodies begin to degenerate around embryonic day (E)14 (Pai, 1965b). Abnormal joint cavitation was reported, and fusion of the cervical and thoracic vertebrae occurred in 40% of newborn *mdg* mice (Pai, 1965a). Development of the neurocranial bones was reported as largely normal, apart from an enlargement of the interparietal and occipal bones. Ribs developed with an abnormal orientation, and were also reported as being thinner in both the calcified and uncalcified regions

at birth than seen in controls. The scapula, clavicle and sternum were found to be decreased in size, and the mandible reduced in length with abnormal curvature in newborn mutant mice (Pai, 1965a). The deltoid tuberosity was absent at birth, and cleft palate was found in 77% of newborn homozygotes (Pai, 1965a).

Many of the features reported for the *mdg* mice have also been observed in ‘muscleless limbs’ mice. Rot-Nivcivic and colleagues (2006) examined skeletal development in double knockouts of *Myf5* and *MyoD*, at E18.5. Many features of the skeleton were affected by the lack of muscle, with fused and enlarged cervical vertebrae, abnormal curvature of the spine and abnormal formation of the mandible, palate, clavicle and sternum (Rot-Nikcevic et al., 2006), as shown in Figure 1. In contrast, normal development of the bones of the neurocranium was observed. Examination of the long bones revealed a shorter tibia and femur and reduced separation between the bones of the zeugopod, but an unchanged length of humerus, radius and ulna compared to normal littermate controls. The humeral tuberosity was reported to be absent at E18.5 and the shapes of the femur and scapula were also affected by the lack of muscle (Rot-Nikcevic et al., 2006). Normal development of the hand and foot bones is also reported (Rot-Nikcevic et al., 2006). In a follow up study (Rot-Nikcevic et al., 2007), the group examined the mandible and clavicle in the muscleless mice, and found that although the shape and size of both skeletal elements were affected in the mutant mice, the clavicle was more dependent on the presence of skeletal muscle than the mandible. In the following year, another study of skeletal development of *Myf5*<sup>-/-</sup>:*MyoD*<sup>-/-</sup> mutant at E18.5 was published by a different group (Gomez et al., 2007). Less mineralization of the femur was found in muscleless mutants, while no difference in bone formation was observed in the phalanx. Changes in shape and diameter were seen in the femur and humerus, but no change in diameter was found in the ulna and radius of mutant animals compared to littermate controls (Gomez et al., 2007). The study also found an unchanged length of humerus and radius, but in contrast to the study of Rot-Nivcivic and colleagues (2006), Gomez and colleagues (2007) reported that the humeral tuberosity was present, although difficult to detect and significantly reduced in size.

A study from our laboratory focussed on the initiation and progression of ossification sites of long bones in the absence of skeletal muscle, which we had shown to be altered in the immobilised chick tibiotarsus (Nowlan et al., 2008b). Therefore, we focussed on an earlier stage of development than previously examined, Theiler Stage (TS) 23 (E14.5). Ossification sites were

found to be differentially affected by the lack of muscle in two mutants; *Myf5<sup>nlacZ/nlacZ</sup>:MyoD<sup>-/-</sup>* and *Pax3<sup>Sp/Sp</sup>* (*Splotch*) (Nowlan et al., 2010). Decreased bone formation was found in the scapula, humerus, ulna and femur but not in the tibia of muscleless limbs. The scapula and humerus were the most severely affected in the muscleless mutants, with ossification centres exhibiting abnormal morphology or even being absent altogether, as shown in Figure 2 (Nowlan et al., 2010). In mice in which muscle mass was reduced by between 35–55% (Rudnicki et al., 1993), bone formation was reduced in comparison to normal littermate controls in the scapula and humerus, while ossification in the ulna, femur and tibia showed no significant differences between reduced muscle mutants and controls (Nowlan et al., 2010). Like the study of Gomez and colleagues (2007), we also found that the humeral tuberosity was present with reduced size in the *Myf5<sup>nlacZ/nlacZ</sup>:MyoD<sup>-/-</sup>* mutants at TS23 (Nowlan et al., 2010). However, in the *Pax3<sup>Sp/Sp</sup>* (*Splotch*) mutants, although the shape of the humeral tuberosity was altered in muscleless limbs, the size at TS23 was not significantly reduced compared to littermate controls (Nowlan et al., 2010). Blitz et al. (2009) showed that the initiation of the deltoid tuberosity is dependent on the presence of tendon, and it has been shown that tendons initiate but are not maintained in the absence of muscle (Brent et al., 2005; Kardon, 1998).

## Joint Development

There are three main stages in joint development, interzone formation, cavitation and morphogenesis, as described in detail in recent reviews (Archer et al., 2003; Pacifici et al., 2005; Pitsillides and Ashhurst, 2008). The detailed shape features of the developing knee joint emerge just following the initiation of muscle contractions in the chick (Roddy et al., 2009). Numerous studies have shown that mechanical forces due to muscle contractions are not critical to the determination of the joint site or to the formation of the interzone (Drachman and Sokóloff, 1966; Kahn et al., 2009; Mikic et al., 2000b; Mitrovic, 1982), whereas the importance of muscle contractions for joint cavitation is indisputable due to the supporting body of evidence as detailed below. In 1966, an elegant study from Drachman and Sokóloff studied the effect of paralysis on joint formation in the chick, using three immobilization methods in order to separate the potential effects of the individual treatments on joint development. Two types of neuromuscular blocking agents were used; decamethonium bromide and type A botulinum toxin, and one surgical method, where extirpation of the lumbosacral cord causes paralysis of the lower

limbs. Drug treated animals (regardless of which drug was administered) exhibited absent or minimal cavitation of the knee, ankle and toe joints (Drachman and Sokóloff, 1966). The plantar tarsal sesamoid was abnormal or absent, while the patella was present in most specimens at day 11 but with reduced size (Drachman and Sokóloff, 1966). Joints of the surgically immobilised animals exhibited similar effects to the drug treated animals, but with more variability between joints and between specimens, and the authors suggest that the passive movement induced in the hindlimbs imparted by normal movement of the upper body led to the range of effects seen (Drachman and Sokóloff, 1966). Subsequent immobilization studies showed that the lack of muscle contractions in the chick prevents cavitation of the hip (Ruano-Gil et al., 1978), knee/femorotibial joint (Osborne et al., 2002; Roddy et al., in preparation; Ruano-Gil et al., 1978), ankle/tibiotarsal joint (Osborne et al., 2002; Persson, 1983), shoulder (Ruano-Gil et al., 1978), elbow (Ruano-Gil et al., 1978), the joints of the hands and feet (Ruano-Gil et al., 1978), the interphalangeal joints (Mitrovic, 1982) and metatarsophalangeal joints (Mitrovic, 1982; Osborne et al., 2002), and the joints between the sternal and vertebral ribs (Hosseini and Hogg, 1991a). Immobilization was found to prevent cavitation of the mobile articulations of the head, cervical spine, larynx and trachea (Murray and Drachman, 1969). Of the 64 joints examined in the head and neck by Murray and Drachman (1969), only three underwent cavitation; two pterygo-parasphenoid joints and one pterygo-palatine joint. In contrast, the sutures of the cranial vault have been found to be largely unaffected by immobilization (Persson, 1983), and the authors conclude that embryonic movements are essential for formation of synovial joints but not for the suture sites of cranial bones (Persson, 1983).

It has been shown that if embryos are immobilised after the joint cavity has formed, the cavity is not fully maintained (Mitrovic, 1982). If movement is increased pharmacologically, wider joint cavities form, as was shown for the hip, shoulder, knee, and the hands and feet (Ruano-Gil et al., 1985). When chicks are paralysed after cavitation has occurred, flaccid paralysis, where muscles are relaxed, has been shown to have a different effect to rigid paralysis (Osborne et al., 2002). Osborne et al., (2002) found that rigid paralysis (using decamethonium bromide) and flaccid paralysis (using pancuronium bromide) both prevent cavitation (of the knee, ankle and metatarsophalangeal joints) if animals are treated prior to initiation of the cavity. However, if animals were immobilised after joint cavities had formed, rigid paralysis partially maintained the cavities and preserved some of the hyaluronan (HA) content in articular surfaces,

while flaccid paralysis led to loss of the cavities and marked reduction in HA content (Osborne et al., 2002). Morphological abnormalities due to immobilization were associated with changes in extracellular matrix molecules by Mikic et al. (2000b), who found altered patterns of tenascin-C and collagen-XII in immobilised foot and femorotibial joints. A mechano-dependant role for extracellular-regulated kinase 1/2 (ERK1/2) was identified by Bastow et al. (2005) who found that ERK1/2 expression was diminished in immobilised limbs, followed by abnormal HA-rich matrix assembly and cavitation-failure (Bastow et al., 2005). Kavanagh et al., (2006) compared the expression patterns of three regulatory factors in the tibiotarsal joints between control and immobilised limbs; FGF-2, FGF-4 and GDF-5, and found that only the expression of FGF-2 in the joint line was altered by immobilization (Kavanagh et al., 2006). The authors propose a mechanoregulatory role for FGF-2 (Kavanagh et al., 2006). Structures associated with the joint are also affected when muscle contractions are removed; Mikic et al. (2000a) examined development of the meniscus of the tibiofemoral joint and the plantar tarsal sesamoid of the tibiotarsal joint in immobilised chicks. A meniscal condensation was evident at day 8 in immobilised embryos, but started to degenerate by day 10 and disappeared by day 11 or 12, while in contrast, the plantar tarsal sesamoid completely failed to form (Mikic et al., 2000a). The authors conclude that mechanical loading is essential for sesamoid formation, while early stages of meniscal formation are intrinsically regulated with mechanical loads required for later stages of meniscal development and maintenance (Mikic et al., 2000a).

The role of mechanical forces in the control of joint shape has been less widely examined than joint cavitation (Roddy et al., 2009a), but a number of studies have indicated that later stages of joint morphogenesis are influenced by the mechanical environment. Our group has found that the abnormal mechanical environment induced by immobilization in the chick has a significant effect on several features of the knee joint such as the height of the lateral and medial condyles of the femur, and width and height of the intercondylar fossa (Roddy et al., 2009b; Roddy et al., in preparation). The overall effect of immobilization is a simpler shape of knee joint with flattened surfaces and absent functional outgrowths (Roddy et al., 2009b; Roddy et al., in preparation).. Flattened articular surfaces have also been reported in the ankle (Drachman and Sokóloff, 1966; Wong et al., 1993), and at the ends of the long bones in paralysed chick embryos (Hosseini and Hogg, 1991a). Computational methods have been used as a means of gaining a more complete understanding of how mechanical forces shape the joint; our laboratory has used

finite element analysis to characterise the biophysical environment of the developing joint, and we have found an association between joint shape, cell proliferation and patterns of biophysical stimuli (Roddy et al., under review). The patella and articular cartilages also develop in regions of dynamic patterns of biophysical stimuli in the interzone (Roddy et al., under review). Heegaard et al. (1999) used a computational model to examine how the emergence of joint shape may be influenced by mechanical forces due to muscle contractions. Inclusion of mechanobiological growth rules led to a more convex-concave articulation of the joint than with a baseline growth rate alone (Heegaard et al., 1999). Changes in the tissue properties of immobilised joints may contribute to the changes in shape, and it has been shown that the mechanical properties, GAG (proteoglycan) and total collagen content of joint epiphyses are affected by immobilization, with a significantly lower instantaneous modulus, GAG content and total collagen content in the upper and lower regions of cartilage cones of immobilised knee joints (Mikic et al., 2004).

While the majority of studies of the mechanics of joint formation *in vivo* have been performed using the chick model (as described above), a small number of studies on mammalian joint development in an abnormal mechanical environment have been published. Abnormal joint contractures were reported for rat embryos immobilised *in utero* with daily injections of D-Tubocurarine (Rodriguez et al., 1992) and for rat embryos in which movement was reduced by daily removal of amniotic fluid (Palacios et al., 1992), but cavitation or morphogenesis of the joints were not examined in detail in either study. Genetically modified ‘muscleless’ mouse models have shown that, while some joints are severely affected by the lack of muscle contractions, other joints form apparently normally (Kahn et al., 2009; Nowlan et al., 2010). Kahn et al. (2009), examined joint formation in three muscleless mouse mutants (*Myf5<sup>-/-</sup>MyoD<sup>-/-</sup>*, splotch delayed mutation (*Sp<sup>d</sup>*) and *Six1<sup>-/-</sup>Six4<sup>-/-</sup>*) and also the *mdg* mutant, in which muscle forms but does not contract, and subsequently degenerates (Pai, 1965b). In the muscleless mice, failure of joint formation in the elbow and shoulder, some carpal elements (capitates to hamate and lunate to triangular), ankle (between talus and calcaneus), some of the metacarpals (carpals, lesser multangular and centrale) and hip was observed (Kahn et al., 2009). No effect was seen in the knee or in the finger joints (Kahn et al., 2009). In the elbows of *Sp<sup>d</sup>* mutants, the interzone forms normally at E12.5 but, in the absence of muscle contractions, the joint progenitor cells do not follow their intended fate and differentiate into chondrocytes (Kahn et al., 2009). Abnormal

$\beta$ -catenin activation was seen in the elbow of E13.5-E14.5  $Sp^d$  mutants but not in the  $Sp^d$  finger joints, and the authors propose that the canonical/ $\beta$ -catenin dependent Wnt pathway may be activated by muscle contractions, modulating joint formation (Kahn et al., 2009). Our laboratory also found abnormal joint development in the elbow, as shown in Figure 2, and shoulder, but normal cavitation of the knee in  $Myf5^{nlacZ/nlacZ};MyoD^{-/-}$  and  $Pax3^{Sp/Sp}$  (*Splotch*) muscleless mutants at E14.5 (Nowlan et al., 2010). However, contrary to the findings of Kahn et al. of an abnormal hip joint in the *mdg* mice at E18.5, we have observed normal cavitation of the hip joint in  $Myf5^{nlacZ/nlacZ};MyoD^{-/-}$  and  $Pax3^{Sp/Sp}$  (*Splotch*) mice at earlier stages (unpublished data), which may reflect a stage-dependant effect or a difference between the model systems used.

## Perspective

Developmental model systems with abnormal skeletal muscle have demonstrated the vital role that mechanical forces play in bone and joint formation. However, we still have very little concrete information on the biological mechanisms that integrate biophysical stimuli with gene regulation. Although cell culture work has revealed long lists of genes responding to mechanical stimulation, demonstration of gene mechano-responsiveness in specific *in vivo* contexts is limited to the studies reviewed in this article (Bastow et al., 2005; Kahn et al., 2009; Kavanagh et al., 2006; Nowlan et al., 2008b; Roddy et al., in preparation) and it is not known if these genes respond directly or indirectly to mechanical stimulation. Kahn et al. (2009) have opened an interesting possibility that Wnt signalling pathways may be mechanically regulated during the formation of some joints, but the cellular pathways that might interpret these mechanical signals are unclear. Animal models can be further exploited to address this question, particularly making use of elegant genetic approaches in the mouse such as lines that report pathway activity crossed with lines showing a muscle phenotype, as used by Kahn et al. (2009). Introduction of reporter and expression constructs into the embryo using *in ovo* electroporation (Nakamura et al., 2004), combined with live imaging, could facilitate more informative manipulation in the chick system. Cell culture work will also be necessary to dissect the cellular mechanisms involved in transducing and integrating mechanical signals with molecular responses. A greater understanding of these mechanisms could enable tissue engineering of more mechanically competent cartilage and bone tissue for replacement therapies.

Despite our limited understanding of the biological mechanisms underlying the mechanobiology of skeletal development, animal models have demonstrated the clear and diverse effects of an altered mechanical environment. We summarise the effects on bone and joint formation in genetically modified mice in which skeletal muscle is absent or non-functional in Figure 3, and chick embryos in which muscle contractions are removed by surgical or pharmacological means on bone and joint development in Figure 4. Rudiments and joints which have been shown to be affected by the absence of muscle, or non-contracting muscle are highlighted in red, while unaffected regions are highlighted in green. Regions for which affected and unaffected aspects have been published, often at different stages of development, are striped red and green. In the chick, all published accounts of immobilization have reported an effect on bone or joint development, with no record of an unaffected aspect (apart from the sutures of the cranial vault, Persson, 1983), while in the mouse, there are several rudiments and joints that have been shown to be unaffected by the absence of muscle, such as the knee joint (Kahn et al., 2009; Nowlan et al., 2010) and the bone of the tibia (Nowlan et al., 2010). An important question to address is why the bones and joints of the mouse seem to be differentially affected by the lack of or alteration in skeletal muscle, while those of the chick are not. Perhaps the mechanical environment external to the embryo itself may have an effect. The chick and mouse have dramatically different mechanical environments during embryonic and foetal development; while the mouse may be affected by movements of the mother and littermates, the only external mechanical stimulus that the chick embryo is subject to is the intermittent rotation of the egg by the mother. We propose that, in muscleless mice, the extrinsic stimulation that the animals experience from movements of the mother and those of normal littermates impact on skeletal development, and that the displacement of the muscleless limbs can affect some parts of the limbs more than others. In as yet unpublished work, our laboratory has used finite element analysis to compute the biophysical stimuli induced by an external displacement applied to the ends of the fore- and hind-limbs, and compare the levels between the humerus (which is more severely affected by absent or reduced muscle at TS23) and the femur (which is less severely affected). The results indicate that the same displacement applied to the ends of the fore-limb and hind-limb induces higher biophysical stimuli in the femur than in the humerus, indicating that the differential effects seen in mammalian models with abnormal skeletal muscle development could be due to external mechanical forces. Variations in the amount of external mechanical



stimulation between embryos would also explain why a range of effects have been seen in muscleless mice; why, for example, some mutants have no bone in the scapular blade at TS23 while, in others, bone is present but does not extend completely across the width of the blade as occurs in controls (Nowlan et al., 2010). This finding could have implications for clinical conditions in which foetal movement is diminished, as it would imply that externally applied stimulation could reduce the severity of the phenotype that results from diminished mechanical forces *in utero*. Additionally, it could provide an interesting insight into the parallel evolution of the integration of mechanical signals in two lineages, where skeletal development in the externally laid, hard shelled bird egg is largely reliant upon intrinsic movement for mechanical stimulation, while the *in-utero* development of the mammal provides sources of external stimulation. This may mean that the mouse is a better model system for human skeletogenesis due to the similar external mechanical environment during development.

To conclude, a range of human conditions in which foetal movement is diminished can impact severely on skeletal development. Animal models which are genetically modified to alter skeletal muscle development, or in which muscle contractions are prevented, demonstrate a clear effect on the lengths of skeletal rudiments, the ossification of rudiments and on joint cavitation and morphogenesis. The most commonly used animal systems are the chick and the mouse, but only the mouse exhibits a clear differential effect of absent or reduced muscle on both bones and joints. We propose that extrinsic mechanical forces impact on skeletogenesis in mammalian models, which could have consequences for minimising the impact of human disorders of reduced movement *in utero*.

## **Acknowledgements**

NN is funded by an intra-European fellowship (IEF) from the Marie Curie Actions under the European Commission Seventh Framework Programme. Unpublished work cited was funded by the Wellcome Trust (083539/Z/07/Z) and by a fellowship to NN from the Irish Research Council for Science Council for Science, Engineering and Technology.

Table 1. Mouse genetic lesions leading to skeletal muscle phenotype used for examination of skeletal development (left), and most commonly employed methods of immobilisation in the chick embryo (right). See text for references.

Mouse Genetic Lesions		Chick Immobilisation Techniques	
Genotype	Effect	Treatment	Effect
<i>Pax3</i> <sup>Sp/Sp</sup> ( <i>Spotch</i> )	Muscleless limbs	Decamethonium bromide	Rigid paralysis
<i>Myf5</i> <sup>nlacZ/nlacZ</sup> : <i>MyoD</i> <sup>-/-</sup>	Muscleless limbs	Pancuronium bromide	Flaccid paralysis
<i>Myf5</i> <sup>nlacZ/+</sup> : <i>MyoD</i> <sup>-/-</sup>	Reduced muscle	Reserpine	Hypermotility
Muscular dysgenesis ( <i>mdg/mdg</i> )	Non-contractile muscle	Surgical techniques	Region-specific paralysis

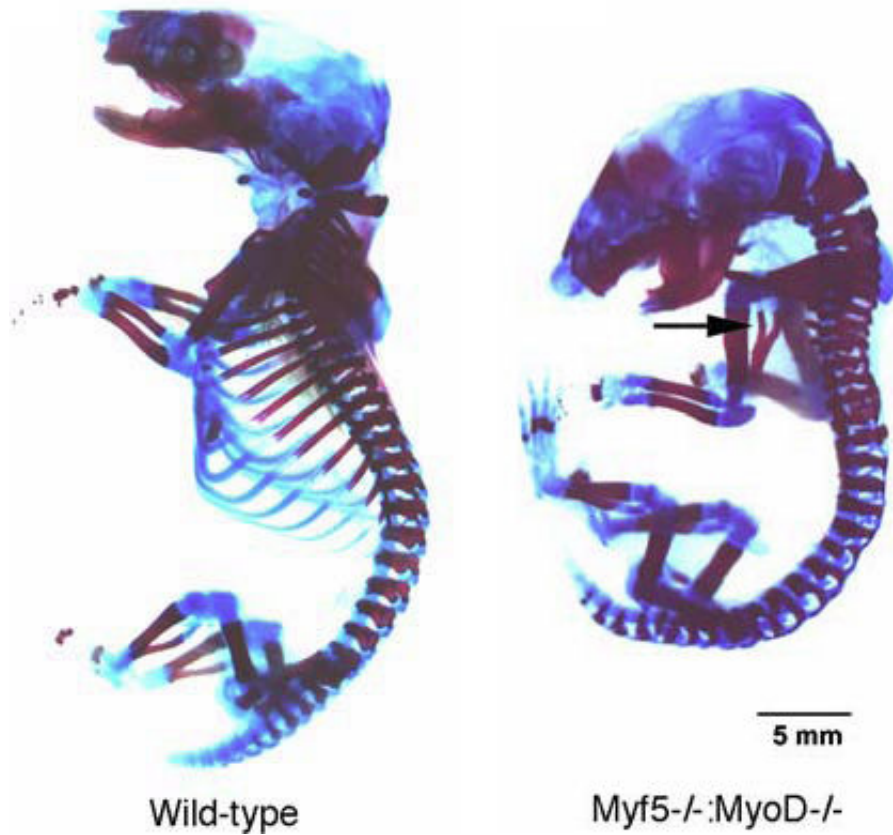


Figure 1. Adapted from Rot-Nikcevic et al., 2006 (with kind permission from Springer Science+Business Media). Skeletal development at E18.5 of wild-type (left), and *Myf5*<sup>-/-</sup>:*MyoD*<sup>-/-</sup> (right), which has no skeletal muscle. Amyogenic mouse have fused and enlarged cervical vertebrae, abnormal curvature of the spine and abnormal formation of the sternum (arrow), mandible, palate and clavicle.

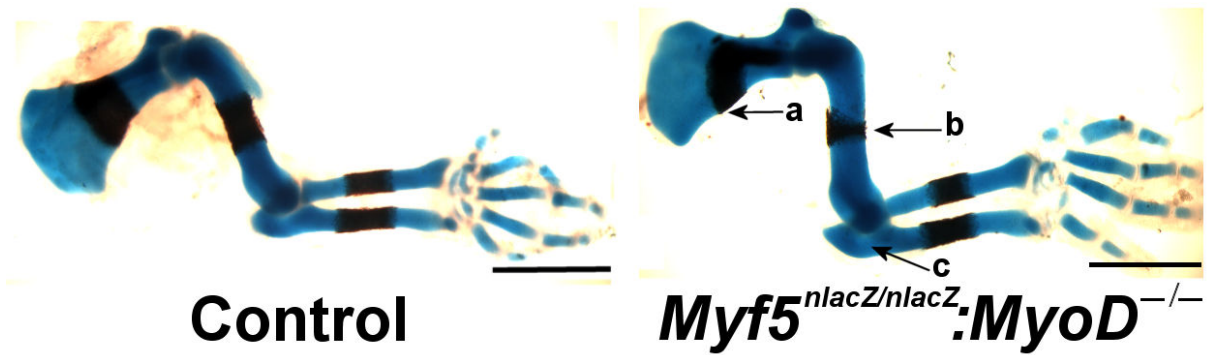


Figure 2. Control (left) and muscleless mutant *Myf5<sup>nlacZ/nlacZ</sup>; MyoD<sup>-/-</sup>* (right) forelimbs at TS23. Effects of absent musculature on forelimb development include incomplete bone formation in scapular blade (a), abnormal morphology of humeral ossification centers (b) and non cavitation of elbow joint (c). Adapted from Nowlan et al. (2010), reproduced with permission from Elsevier.



Figure 3. Summary of effects seen in mouse models with an abnormal muscle development shown in cartoon of TS24 mouse skeleton. Red indicates effect on rudiment or joint due to abnormal muscle, green indicates no effect, striped red and green indicates findings of affected and unaffected aspects as detailed below, and white indicates no data available. Rudiments: cervical vertebrae [1, 3], scapular length [1–3] scapular bone [2], scapular spine bone [2], clavicle [1, 3, 4], sternum [1, 3], ribs [3], humeral length [1, 5: unaffected at e18.5, 2: affected at TS23], humeral bone [2], humeral tuberosity [1–3], ulnar length [1: unaffected at e18.5, 2: affected at TS23] ulnar bone [2: affected at TS23], radius length [1, 5], hand rudiments [1], femoral length [1: affected at e18.5, 2: affected in TS23 *Myf5<sup>nlacZ/nlacZ</sup>:MyoD<sup>-/-</sup>*, unaffected in TS23 *Pax3<sup>Sp/Sp</sup>*], femoral bone [2, 5], tibial length [1: affected at e18.5, 2: affected in TS23 *Pax3<sup>Sp/Sp</sup>*, 2: unaffected in TS23 *Myf5<sup>nlacZ/nlacZ</sup>:MyoD<sup>-/-</sup>*], tibial bone [2: unaffected], foot rudiments [1], second phalanx ossification [5]. Joints: cervical vertebrae [1, 3, 5, 6], thoracic vertebrae [3], lumbar vertebrae [6], shoulder [2, 3, 6] elbow [2, 6] carpal joints [6], finger joints [6], hip [6: affected, unpublished data: unaffected], knee [2, 6], talus joints [6], metacarpal joints [6]. Also affected but not shown: mandible [1, 3, 4], palate [3], curvature of spine [1, 3]. [1] Rot-Nikcevic et al., 2006; [2] Nowlan et al., 2010; [3] Pai, 1965a; [4] Rot-Nikcevic et al., 2007; [5] Gomez et al., 2007; [6] Kahn et al., 2009.

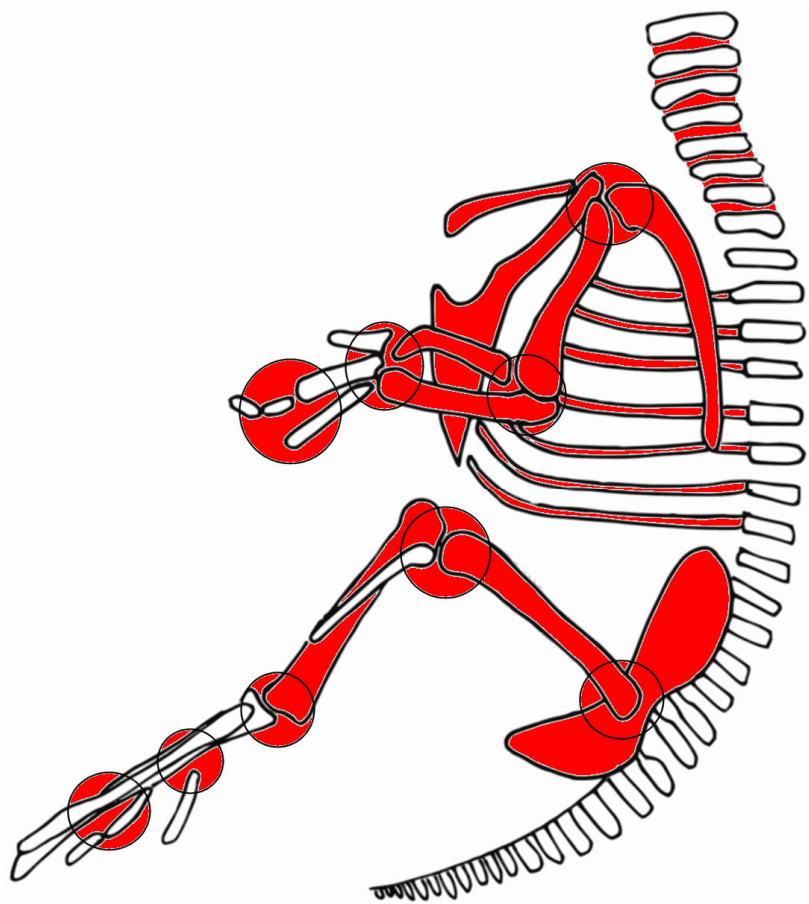


Figure 4. Summary of effects seen in immobilised chick models shown in cartoon of HH34 chick skeleton. Red indicates effect on rudiment or joint due to abnormal muscle and white indicates no data available. Rudiments: scapula [1], clavicle [2], sternum [2], ribs [2], humerus [1, 2], ulna [1], radius [1], pelvic girdle [1], femur [1, 2, 4], tibiotarsus [1-6]. Joints: cervical spine [12], shoulder [7], elbow [7], metatarsophalangeal [9-11], interphalangeal [7, 11], hip [7], knee [7-9, 13-15, 18, 19], ankle [8-10, 17], feet [7, 8, 13]. Affected but not shown: lower beak [2, 12], curvature of neck and spine [2], mandible [2], joints between sterna and vertebral ribs [1], articulations of head and lower jaw, larynx and trachea [12], plantar tarsal sesamoid [8, 16] and meniscus of tibiofemoral joint [16]. Unaffected and not shown: sutures of cranial vault [10].

[1] Hosseini and Hogg, 1991a; [2] Hall and Herring, 1990; [3] Hosseini and Hogg, 1991b, [4] Wong et al., 1993; [5] Germiller and Goldstein, 1997; [6] Nowlan et al., 2008b; [7] Ruano-Gil et al., 1978; [8] Drachman and Sokóloff, 1966; [9] Osborne et al., 2002; [10] Persson et al., 1983; [11] Mitrovic, 1982; [12] Murray and Drachman, 1966; [13] Mikic et al., 2000b; [14] Bastow et al., 2005; [15] Kavanagh et al., 2006; [16] Mikic et al., 2000a; [17] Wong et al., 1993; [18] Mikic et al., 2004; [19] Roddy et al., in preparation.

## References

- Archer CW, Dowthwaite GP, Francis-West P. 2003. Development of synovial joints. *Birth Defects Res C Embryo Today* 69(2):144-155.
- Bastow ER, Lamb KJ, Lewthwaite JC, Osborne AC, Kavanagh E, Wheeler-Jones CP, Pitsillides AA. 2005. Selective activation of the MEK-ERK pathway is regulated by mechanical stimuli in forming joints and promotes pericellular matrix formation. *J Biol Chem* 280(12):11749-11758.
- Blitz E, Viukov S, Sharir A, Shwartz Y, Galloway J, Pryce B, Johnson R, Tabin C, Schweitzer R, Zelzer E. 2009. Bone Ridge Patterning during Musculoskeletal Assembly Is Mediated through SCX Regulation of Bmp4 at the Tendon-Skeleton Junction. *Developmental cell* 17(6):861-873.
- Blumer MJ, Longato S, Richter E, Perez MT, Konakci KZ, Fritsch H. 2005. The role of cartilage canals in endochondral and perichondral bone formation: are there similarities between these two processes? *J Anat* 206(4):359-372.
- Brent A, Braun T, Tabin C. 2005. Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. *Development* 132(3):515.
- Dabezies E, Warren P. 1997. Fractures in very low birth weight infants with rickets. *Clinical orthopaedics and related research* 335:233.
- Doménech-Ratto G, Fernández-Villacanas Marín M, Ballester-Moreno A, Doménech-Asensi P. 1999. Development and segments of cartilage canals in the chick embryo. *Eur J Anat* 3:121-126.
- Drachman DB, Sokóloff L. 1966. The role of movement in embryonic joint formation. *Developmental Biology* 14:401-420.
- Fell HB. 1925. The histogenesis of cartilage and bone in the long bones of the embryonic fowl. *Journal of Morphology and Physiology* 40(3):417-459.
- Fell HB, Canti RG. 1934. Experiments on the development *in vitro* of the avian knee joint. *Proc Roy Soc B* 116:316-351.
- Franz T, Kothary R, Surani MAH, Halata Z, Grim M. 1993. The Splotch Mutation Interferes With Muscle Development In The Limbs. *Anatomy and Embryology* 187(2):153-160.
- Germiller JA, Goldstein SA. 1997. Structure and function of embryonic growth plate in the absence of functioning skeletal muscle. *J Orthop Res* 15(3):362-370.
- Gomez C, David V, Peet NM, Vico L, Chenu C, Malaval L, Skerry TM. 2007. Absence of mechanical loading in utero influences bone mass and architecture but not innervation in Myod-Myf5-deficient mice. *J Anat* 210(3):259-271.
- Gould SJ. 1985. *Ontogeny and Phylogeny*. Cambridge: Belknap Press of Harvard University Press.
- Hall BK. 1972. Immobilization and cartilage transformation into bone in the embryonic chick. *The Anatomical Record* 173(4):391-403.
- Hall BK. 1987. Earliest evidence of cartilage and bone development in embryonic life. *Clin Orthop Relat Res* 225:255-272.
- Hall BK, Herring SW. 1990. Paralysis and growth of the musculoskeletal system in the embryonic chick. *J Morphol* 206(1):45-56.
- Hall JG. 1986. Analysis of Pena Shokeir phenotype. *American journal of medical genetics* 25(1):99.
- Ham AW, Cormack DH. 1979. *Histophysiology of Cartilage, Bone and Joints*. Philadelphia: J.B. Lippincott.
- Hammond E, Donnenfeld AE. 1995. Fetal Akinesia. *Obstetrical & Gynecological Survey* 50(3):240-249.
- Heegaard JH, Beaupré GS, Carter DR. 1999. Mechanically modulated cartilage growth may regulate joint surface morphogenesis. *J Orthop Res* 17(4):509-517.
- Henderson JH, Carter DR. 2002. Mechanical induction in limb morphogenesis: the role of growth-generated strains and pressures. *Bone* 31(6):645-653.
- His W. 1888. On the principles of animal morphology. *Proc Roy Soc Edinburgh* 15:287-298.
- Hogg DA. 1980. A re-investigation of the centres of ossification in the avian skeleton at and after hatching. *J Anat* 130(Pt 4):725-743.

- Homer C, Baltz R, Hickson G, Miles P, Newman T, Shook J, WM Z. 2000. Clinical practice guideline: early detection of developmental dysplasia of the hip. Committee on Quality Improvement, Subcommittee on Developmental Dysplasia of the Hip. American Academy of Pediatrics. *Pediatrics* 105:896-905.
- Hosseini A, Hogg DA. 1991a. The effects of paralysis on skeletal development in the chick embryo. I. General effects. *J Anat* 177:159-168.
- Hosseini A, Hogg DA. 1991b. The effects of paralysis on skeletal development in the chick embryo. II. Effects on histogenesis of the tibia. *J Anat* 177:169-178.
- Ingber DE, Mow VC, Butler D, Niklason L, Huard J, Mao J, Yannas I, Kaplan D, Vunjak-Novakovic G. 2006. Tissue engineering and developmental biology: Going biomimetic. *Tissue Engineering* 12(12):3265-3283.
- Jee WSS. 1988. The skeletal tissues. In: Weiss L, W., editor. *Cell and Tissue Biology*. Baltimore, Munich: Urban & Schwarzenberg.
- Kablar B, Krastel K, Tajbakhsh S, Rudnicki MA. 2003. Myf5 and MyoD activation define independent myogenic compartments during embryonic development. *Developmental Biology* 258(2):307-318.
- Kahn J, Shwartz Y, Blitz E, Krief S, Sharir A, Breitel DA, Rattenbach R, Relaix F, Maire P, Rountree RB, Kingsley DM, Zelzer E. 2009. Muscle contraction is necessary to maintain joint progenitor cell fate. *Development* 136(5):734-743.
- Kardon G. 1998. Muscle and tendon morphogenesis in the avian hind limb. *Development* 125(20):4019-4032.
- Kassar-Duchossoy L, Gayraud-Morel B, Gomes D, Rocancourt D, Buckingham M, Shinin V, Tajbakhsh S. 2004. Mrf4 determines skeletal muscle identity in Myf5 : MyoD double-mutant mice. *Nature* 431(7007):466-471.
- Kavanagh E, Church VL, Osborne AC, Lamb KJ, Archer CW, Francis-West PH, Pitsillides AA. 2006. Differential regulation of GDF-5 and FGF-2/4 by immobilisation in ovo exposes distinct roles in joint formation. *Dev Dyn* 235(3):826-834.
- Kelly D, Jacobs C. 2010. The role of mechanical signals in regulating chondrogenesis and osteogenesis of mesenchymal stem cells. *Birth Defects Research Part C: Embryo Today: Reviews* 90(1):75-85.
- Laurin M, Fradet N, Blangy A, Hall A, Vuori K, Côté J. 2008. The atypical Rac activator Dock180 (Dock1) regulates myoblast fusion in vivo. *Proceedings of the National Academy of Sciences* 105(40):15446.
- McMahon LA, O'Brien FJ, Prendergast PJ. 2008. Biomechanics and mechanobiology in osteochondral tissues. *Regen Med* 3(5):743-759.
- Mikic B, Isenstein AL, Chhabra A. 2004. Mechanical modulation of cartilage structure and function during embryogenesis in the chick. *Ann Biomed Eng* 32(1):18-25.
- Mikic B, Johnson TL, Chhabra AB, Schalet BJ, Wong M, Hunziker EB. 2000a. Differential effects of embryonic immobilization on the development of fibrocartilaginous skeletal elements. *J Rehabil Res Dev* 37(2):127-133.
- Mikic B, Wong M, Chiquet M, Hunziker EB. 2000b. Mechanical modulation of tenascin-C and collagen-XII expression during avian synovial joint formation. *J Orthop Res* 18(3):406-415.
- Miller ME. 2003. The bone disease of preterm birth: a biomechanical perspective. *Pediatr Res* 53(1):10-15.
- Miller ME, Hangartner TN. 1999. Temporary brittle bone disease: association with decreased fetal movement and osteopenia. *Calcif Tissue Int* 64(2):137-143.
- Mitrovic D. 1982. Development of the articular cavity in paralyzed chick embryos and in chick embryo limb buds cultured on chorioallantoic membranes. *Acta Anat (Basel)* 113(4):313-324.
- Moyer-Mileur L, Brunstetter V, McNaught T, Gill G, Chan G. 2000. Daily physical activity program increases bone mineralization and growth in preterm very low birth weight infants. *Pediatrics* 106(5):1088.



- Murray PD, Drachman DB. 1969. The role of movement in the development of joints and related structures: the head and neck in the chick embryo. *J Embryol Exp Morphol* 22(3):349-371.
- Murray PDF, Selby D. 1930. Intrinsic and extrinsic factors in the primary development of the skeleton. *Wilhelm Roux' Arch Entwicklungmech Org* 122:629-662.
- Nakamura H, Katahira T, Sato T, Watanabe Y, Funahashi J. 2004. Gain-and loss-of-function in chick embryos by electroporation. *Mechanisms of Development* 121(9):1137-1143.
- Nicole S, Diaz CC, Frugier T, Melki J. 2002. Spinal muscular atrophy: recent advances and future prospects. *Muscle Nerve* 26(1):4-13.
- Nowlan NC, Bourdon C, Dumas G, Tajbakhsh S, Prendergast PJ, Murphy P. 2010. Developing bones are differentially affected by compromised skeletal muscle formation. *Bone* 46(5):1275-1285.
- Nowlan NC, Murphy P, Prendergast PJ. 2007. Mechanobiology of embryonic limb development. *Ann N Y Acad Sci* 1101:389-411.
- Nowlan NC, Murphy P, Prendergast PJ. 2008a. A dynamic pattern of mechanical stimulation promotes ossification in avian embryonic long bones. *J Biomech* 41(2):249-258.
- Nowlan NC, Prendergast PJ, Murphy P. 2008b. Identification of Mechanosensitive Genes during Embryonic Bone Formation. *PLoS Computational Biology* 4(12):e1000250.
- Osborne AC, Lamb KJ, Lewthwaite JC, Dowthwaite GP, Pitsillides AA. 2002. Short-term rigid and flaccid paralyses diminish growth of embryonic chick limbs and abrogate joint cavity formation but differentially preserve pre-cavitated joints. *J Musculoskelet Neuronal Interact* 2(5):448-456.
- Pacifici M, Koyama E, Iwamoto M. 2005. Mechanisms of synovial joint and articular cartilage formation: recent advances, but many lingering mysteries. *Birth Defects Res C Embryo Today* 75(3):237-248.
- Pai A. 1965a. Developmental genetics of a lethal mutation, muscular dysgenesis (mdg), in the mouse. I. Genetic analysis and gross morphology. *Developmental Biology* 11:82.
- Pai A. 1965b. Developmental genetics of a lethal mutation, muscular dysgenesis (mdg), in the mouse. II. Developmental analysis. *Developmental Biology* 11:93.
- Palacios J, Rodriguez JI, Ruiz A, Sanchez M, Alvarez I, DeMiguel E. 1992. Long bone development in extrinsic fetal akinesia: an experimental study in rat fetuses subjected to oligohydramnios. *Teratology* 46(1):79-84.
- Persson M. 1983. The role of movements in the development of sutural and diarthrodial joints tested by long-term paralysis of chick embryos. *J Anat* 137(Pt 3):591.
- Pitsillides A, Ashhurst D. 2008. A critical evaluation of specific aspects of joint development. *Developmental Dynamics* 237(9):2284-2294.
- Powell J, Petherbridge L, Flucher B. 1996. Formation of triads without the dihydropyridine receptor alpha subunits in cell lines from dysgenic skeletal muscle. *Journal of Cell Biology* 134(2):375.
- Roberts CD, Vogtle L, Stevenson RD. 1994. Effect Of Hemiplegia On Skeletal Maturation. *Journal of Pediatrics* 125(5):824-828.
- Roddy KA, Kelly GM, van Es MH, Murphy P, Prendergast PJ. under review. Dynamic patterns of mechanical stimulation correlate with growth and cell proliferation during morphogenesis in the avian embryonic knee joint.
- Roddy KA, Nowlan NC, Prendergast PJ, Murphy P. 2009a. 3D representation of the developing chick knee joint: a novel approach integrating multiple components. *J Anat* 214(3):374-387.
- Roddy KA, Nowlan NC, Prendergast PJ, Murphy P. 2009b. The Influence of Mechanical Forces on the Development of Shape in the Avian Knee Joint. 55th Annual Meeting of the Orthopaedic Research Society. Las Vegas, Nevada, U.S.A.
- Roddy KA, Prendergast PJ, Murphy P. in preparation. Mechanical influences on morphogenesis of the knee joint revealed through morphological and molecular analysis and computational modelling of immobilised embryos.

- Rodriguez JI, Garcia-Alix A, Palacios J, Paniagua R. 1988a. Changes in the long bones due to fetal immobility caused by neuromuscular disease. A radiographic and histological study. *J Bone Joint Surg Am* 70(7):1052-1060.
- Rodriguez JI, Palacios J, Garcia-Alix A, Pastor I, Paniagua R. 1988b. Effects of immobilization on fetal bone development. A morphometric study in newborns with congenital neuromuscular diseases with intrauterine onset. *Calcif Tissue Int* 43(6):335-339.
- Rodriguez JI, Palacios J, Ruiz A, Sanchez M, Alvarez I, Demiguel E. 1992. Morphological changes in long bone development in fetal akinesia deformation sequence: an experimental study in curarized rat fetuses. *Teratology* 45(2):213-221.
- Rot-Nikcevic I, Downing KJ, Hall BK, Kablar B. 2007. Development of the mouse mandibles and clavicles in the absence of skeletal myogenesis. *Histol Histopathol* 22(1):51-60.
- Rot-Nikcevic I, Reddy T, Downing KJ, Belliveau AC, Hallgrimsson B, Hall BK, Kablar B. 2006. Myf5<sup>-/-</sup>:Myod<sup>-/-</sup> amyogenic fetuses reveal the importance of early contraction and static loading by striated muscle in mouse skeletogenesis. *Dev Genes Evol* 216(1):1-9.
- Roux W. 1894. Einleitung zum Archiv für Entwicklungsmechanik. The problems, methods and scope of developmental mechanics, translated by W. M. Wheeler (in 1894). English translation reprinted in J. M. Maienschein (ed.), *Defining Biology*, 1986. Harvard University Press, Cambridge, MA, pp. 107–148. *Archiv für Entwicklungsmechanik der Organismen* 1:pp. 1-142.
- Ruano-Gil D, Nardi-Villardaga J, Teixidor-Johe A. 1985. Embryonal hypermobility and articular development. *Acta Anat (Basel)* 123(2):90-92.
- Ruano-Gil D, Nardi-Villardaga J, Tejedó-Mateu A. 1978. Influence of extrinsic factors on the development of the articular system. *Acta Anat (Basel)* 101(1):36-44.
- Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R. 1993. Myod or Myf-5 is required for the formation of skeletal muscle. *Cell* 75(7):1351-1359.
- Shefelbine SJ, Carter DR. 2004. Mechanobiological predictions of growth front morphology in developmental hip dysplasia. *J Orthop Res* 22(2):346-352.
- Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M. 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of Myod. *Cell* 89(1):127-138.
- Vanden Berg-Foels WS, Todhunter RJ, Schwager SJ, Reeves AP. 2006. Effect of Early Postnatal Body Weight on Femoral Head Ossification Onset and Hip Osteoarthritis in a Canine Model of Developmental Dysplasia of the Hip. *Pediatric research* 60(5):549-554
- Weinstein S. 1987. Natural history of congenital hip dislocation (CDH) and hip dysplasia. *Clinical orthopaedics and related research* 225:62.
- Wesstrom G, Bensch J, Schollin J. 1986. Congenital Myotonic Dystrophy. *Acta Paediatrica* 75(5):849-854.
- Wong M, Germiller J, Bonadio J, Goldstein SA. 1993. Neuromuscular atrophy alters collagen gene expression, pattern formation, and mechanical integrity of the chick embryo long bone. *Prog Clin Biol Res* 383B:587-597.