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Mechanobiology of Embryonic Limb Development

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ABSTRACT: Considerable evidence exists to support the hypothesis that mechanical forces have an essential influence on embryonic skeletal development. Clinical observations and experimental data indicate the importance of muscle contractions for limb development. However, the influence of these forces is seldom referred to in biological descriptions of bone development, and perhaps this is due to the fact that the hypothesis that mechanical forces are essential for normal embryonic skeletal development is difficult to test and elaborate experimentally *in vivo*, particularly in humans. Computational modeling has the potential to address this issue by simulating embryonic growth under a range of loading conditions but the potential of such models has yet to be fully exploited. In this paper, we review the literature on mechanobiology of limb development in three main sections; a. experimental alteration of the mechanical environment, b. mechanical properties of embryonic tissues and c. the use of computational models. Then we analyze the main issues, and suggest how experimental and computational fields could work closer together to enhance our understanding of mechanobiology of the embryonic skeleton.

INTRODUCTION

To what extent do mechanical forces affect skeletal development in the embryo? As early as the 1920's this question was debated with some researchers holding that muscle contractions had no influence on limb shape[1], whereas others argued that mechanical forces were the primary influence on skeletal growth and development[2]. Now, over 80 years later, the importance of mechanical forces on skeletogenesis in the embryo has been recognized, but it still has not been the subject of much detailed investigation. For example, how mechanical forces may regulate gene expression in the embryonic skeleton has yet to be systematically studied.

A number of rare neuromuscular disorders that cause reduced muscle contractions in the fetus are the primary evidence of the effect of forces on skeletal development in the human. Rodríguez *et al.*[3, 4] describe how fetal immobilization due to congenital myotonic dystrophy or spinal muscular atrophy can have a dramatic effect on the human skeleton, in particular the long bones, which were found to be thin, hypomineralized and elongated, often with multiple diaphyseal or metaphyseal fractures, (Figure 1). Although most fractures occurred during birth and postnatal handling, there was evidence of bone fracture *in utero* in some infants, particularly at the growth plate. The authors attribute the etiology of the fractures and the mechanical properties of the bone to the "reduction in the intrauterine motion of the fetus"[3]. An abnormal mechanical environment *in utero* is also thought to be a factor in developmental dysplasia of the hip, where forces due to limb position, pressure from the womb or ligament laxity lead to altered growth and bony deformities in the neo-natal hip joint[5].

Two types of ossification occur during embryonic skeletal development; intramembranous and endochondral. Intramembranous ossification occurs when bone develops directly from mesenchyme and is found in the flat bones of the skull and face. In

endochondral ossification, bone replaces a pre-existing cartilaginous template[6]. In the mammalian long bone, a cartilaginous template of each rudiment is ossified by a precise series of events. First there is hypertrophy of the chondrocytes in the mid-diaphysis of the rudiment, followed by invasion of the perichondrium by capillaries, and the formation of a periosteal bone collar via intramembranous ossification around the circumference[6, 7]. Then, following invasion by blood vessels, the cartilage core of the rudiment begins to be mineralized, and replaced by bone[6]. This endochondral ossification at the centre of the cartilage and the periosteal bone collar at the circumference are known collectively as the primary ossification centre. Blood vessels and vascular mesenchyme then separate two zones of ossification (growth plates) which progress distally and proximally along the diaphysis of the bone[8], leaving behind internal bone of ossified spicules, which is then remodeled into cancellous bone or resorbed to form the marrow cavity[9] (Fig. 2)[10]. In mammals, secondary ossification centers later develop in the upper and lower epiphyseal cartilage of most long bones. Eventually, the epiphyses are closed when the epiphyseal and metaphyseal trabeculae fuse[8].

Although it has been accepted that mechanical forces are important factors in some aspects of skeletal biology (for example, bone loss in space due to reduced mechanical forces in the absence of gravity[11]), the hypothesis that embryonic bone development is influenced by mechanical forces is mentioned only briefly (if at all) in standard accounts of skeletogenesis. While many genes instrumental in skeletogensis have been identified as being mechanosensitive such as Indian hedgehog (Ihh)[12], parathyroid hormone receptor protein (PTHrP)[13] and members of the bone morphogenic protein (BMP)[14] family, the interaction between these and other mechanosensitive genes and gene products and the processes of embryonic bone development have not yet been characterized.

The embryonic chick has been used extensively to study the influence of muscle contractions on embryonic skeletal growth, as described in the following section on

experimental alteration of the embryonic mechanical environment. As an amniote, the chick shares many features of embryonic development with mammals and has the huge advantage of development external to the mother, allowing procedures and alterations to be performed and the effects on the embryo examined. The chick embryo is also quite resilient, and can withstand procedures such as immobilization and muscle ablation. However, it is seldom acknowledged that long bone development in fowl is significantly different from mammals. Two major differences exist between avian and mammalian skeletal development, (Figure 3). Firstly, avian long bones have no primary (endochondral) ossification centre[15] and secondly, in the embryonic chick, vascularization of the primary cartilage is not present prior to mineralization[6]. In the chick, by 6 to 6.5 days of incubation, chondrocytes in the mid-diaphysis undergo hypertrophy, and by 6.5 to 7 days of incubation, bone collar formation begins in the mid region of the diaphysis with the deposition of osteoid below the perichondrium[6]. The first mineralization takes place 0.5 to 1 days later[6] in the form of laminae of bone which eventually fuse to form a thin, compact cylinder- the periosteal bone collar. This cylinder increases in thickness, and becomes richly vascularized, giving rise to trabeculae[15]. At this point, erosion of the cartilage inside the bone collar begins, and progresses in long finger-like protuberances[16]. As stated by Hall[6], and in contrast to the mammal, the primary cartilage in the chick is never invaded by blood vessels during embryonic life. As ossification continues, the periosteal bone collar advances along the diaphysis to the proximal and distal epiphyses, increasing in thickness by addition of osseous trabeculae and the enlargement of those already formed[15]. According to Fell[15], core endochondral ossification in the chick takes places only in the extremities of the diaphysis, in positions analogous to secondary ossification sites in the mammal; however, the presence or otherwise of secondary ossification centers in the chick (*Gallus gallus)* is still not a settled issue, with several researchers reporting only one

secondary ossification centre in the proximal tibiotarsus[17, 18], and others also identifying one in the distal femur (but not in every specimen examined)[19].

Computational mechanobiology determines the quantitative rules or algorithms that govern the effects of mechanical loading on tissue differentiation, growth, adaptation and maintenance[20, 21]. A computer simulation in mechanobiology has two parts, as described by van der Meulen and Huiskes[20]; a mechanics part, where the boundary forces of a domain are translated into local biophysical stimuli depending on geometry and mechanical properties, usually performed with Finite Element (FE) analysis, and a biological part, where it is hypothesized how mechanical stimuli lead to genetic events or biological processes in the tissue. The mechanical properties of the tissues under investigation are an important component of the FE analysis, and the (somewhat limited) data available on embryonic tissues are reviewed here. Computational mechanobiology involves framing a hypothesis relating mechanical forces to biological changes or events, and designing a computational scheme to test this hypothesis[20]. Computational models can be used in isolation, but are more likely to be accepted by other researchers if corroborated by experimental data. In situations that cannot be tested experimentally, computational models can provide a useful insight into possible influences of mechanical forces.

The following sections review the literature on (a) experimental alterations of the embryonic mechanical environment, (b) the mechanical properties of embryonic tissues, and (c) computational work on embryonic skeletal development, with particular focus on the effects of muscle contractions on long bones in the limb.

EXPERIMENTAL ALTERATION OF THE MECHANICAL ENVIRONMENT IN THE EMBRYO

Experiments where the mechanical environment in the embryo is altered can be broadly grouped into two categories: (i) *in vivo* experiments, mainly immobilization

procedures, but also other surgical procedures such as muscle ablation or partial neural tube excision. In *in vivo* immobilization studies, embryonic muscle contractions are prevented by the application of neuro-muscular blocking agents. (ii) *ex vivo*, comprising *in vitro* and grafting experiments. *In vitro* experiments involve removing the limb (or part thereof) from an embryo and growing it in culture. Numerous aspects of skeletal development have been examined by culturing embryonic rudiments *in vitro*, e.g. such as patterning and shape development[22], ossification (both periosteal[23] and endochondral[24-26]), secondary cartilage[27, 28] and joint formation[29, 30]. Grafting experiments also involve separation of the limb or skeletal rudiments from the embryo, and are among the most commonly performed scientific procedures in the chick. In a limb graft, the test limb can be attached to the coelemic cavity of a "host embryo", or, for a chorioallantoic membrane graft, the graft is implanted into the chorio-allantois of the host – a thin vascular membrane extending from the embryo. The grafted structure (e.g., femur) receives nourishment and oxygen from the bloodstream of the host, but probably no innervation, according to Murray and Selby[16]. Assuming all muscle tissue is removed prior to grafting, growth in the absence of muscle contractions can then be examined. However, as with all of the above experimental procedures, forces due to growth related strains or pressures [31-34] or forces due to the surrounding environment [16] are *not* eliminated.

Shape

In vitro [35, 36] and chorioallantoic graft[16] studies have both indicated that shape and growth of the early cartilaginous skeletal long bone rudiments can proceed fairly normally in the absence of muscle contractions with the following exceptions. In the case of chorioallantoic grafts of the femur[16], smaller articular structures and reduced inter-condyloid fossa were found, and the groove across the head of the femur, which normally holds the acetabular ligament, was missing. Abnormal curvature of the rudiments grown *in*

vitro was found by Murray and Selby [16] and also by Thorogood [31]. Long and Lisenmayer[37] examined the role of the perichondrium using an organ culture system of chicken embryonic tibiotarsi, where growth in the absence of a perichondrium was compared to that of controls. The perichondiumm-free tibiotarsi exhibited a higher growth rate, and based on their findings, the authors suggest that the presence of the perichondrium negatively regulates hypertrophy and proliferation of chondrocytes.

Ossification

The literature describing experimental work on the mechanobiology of embryonic ossification is disjointed, with a range of (sometimes sparse) data on different species, types of ossification, bone locations and embryonic ages. It has been shown that periosteal ossification in chick long bones will occur in grafts[16] and in culture[23], and endochondral ossification will occur in murine long bone[25, 26] and pubic bone[24] rudiments in culture. Periosteal ossification does not proceed in a normal fashion in a chorio-allantoic graft of the embryonic avian femur (6 or 7 days of incubation)[16] with greater deposition of bone on the concave curve of the bone compared to a normal avian femur. Glucksman[23, 38] cultured embryonic chick bone rudiments (from 7 to 13 days incubation) in such a position that they exerted pressure on each other as they grew. The author found that increased tension increased the rate of bone formation while reduced tension diminished ossification, and concluded that mechanical stress stimulates osteogenesis *in vitro*. Klein-Nulend *et al.*[25] cultured embryonic mouse long bone rudiments aged embryonic day 16 (E16) *in vitro* and examined the effect on calcification of intermittently or continuously compressing the gas phase above the culture medium. The authors found that the compressive force resulted in increased calcification of the growth plate cartilage, with intermittent forces having a greater effect than continuous forces, and concluded that mechanical loading seems to be an important regulator of biomineralization[25]. Tanck *et al.*[26] cultured embryonic mouse

metatarsal rudiments at E15 and E17 *in vitro*, and compared the geometry of the primary ossification centers of the cultured rudiments to those of controls. The authors found that the mineralization front *in vivo* was almost straight, but was convex for *in vitro* grown rudiments, as shown in Figure 4.

Endochondral ossification *in vitro* was observed in mouse pubic bone rami rudiments excised at E13, and in cultures of human embryonic mandibular condyles[39]. Felts[40] in 1959 implanted mouse and rat post-natal (2 & 5 days) long bones subcutaneously into a litter mate or adult of the same species, and claims to have observed normal ossification patterns and largely normal shape development of the rudiments. He concludes that long bone size, shape and ossification events are independent of the mechanical environment, although the author never attempted to quantify the mechanical environment of the subcutaneous implant. It is also important to note that ossification occurring *ex vivo* could be due to prior commitment of the cells towards a certain developmental fate[31]; therefore the timing of the manipulation may be of crucial importance and just because ossification was observed in grafted or cultured limbs does not mean cartilage differentiation is entirely independent of mechanical environment. Although the study of Sundaramurthy and Mao[41] was performed on neo-natal rather than embryonic skeletal elements, it is included in this review because it demonstrates modulation of secondary ossification centre formation by mechanical loading. The authors found that when distal femoral condyle explants from neonatal rabbits were submitted to cyclic loading, a structure reminiscent of the secondary ossification centre appeared whereas no secondary ossification centre was detected in any of the unloaded control specimens. The mechanically loaded specimens expressed Runx2, osteopontin and Type X collagen, which were absent in the unloaded controls, and loaded specimens also had a significantly higher number of hypertrophic chondrocytes than controls. The authors conclude that mechanical stresses accelerate the formation of the secondary ossification centre, and therefore modulate endochondral ossification.

Joint Formation

Joint formation is one aspect of skeletal embryogenesis commonly acknowledged to be strongly influenced by mechanical forces, specifically those forces due to muscle contractions *in ovo/ in utero*. Formation of a typical diarthrodial embryonic joint takes place in two stages; firstly, the joint region becomes recognizable as three layers; an interzone and the two future epiphyseal surfaces, and secondly, the joint cavity is formed[31]. The first stage occurs *in vitro*[30, 42], in chorioallantoic grafts[43] and in embryos immobilized using neuro-muscular blocking agents[44, 45], however, mechanical stimulation is needed for cavitation[31, 44, 45]. Rudiments cultured *in vitro* result in cartilaginous fusion of the opposed joint elements, unless the limbs are artificially stimulated, in which case fusion does not occur [30], as shown in Figure 5.

Mikic *et al.* [44] treated chick embryos *in ovo* with a neuromuscular blocking agent and found partial or absent cavitation, and post-cavitational joint fusion if the drug was applied after initial cavitation occurred. Osborne *et al.*[45] examined joint formation in the chick with two different methods of immobilization; rigid paralysis (dynamic stimulation removed) and flaccid paralysis (static and dynamic stimulation removed). Immobilization using either method before and during the time of normal cavitation of joints resulted in loss of cavitation. If immobilization was induced after a cavity had arisen, loss of cavitation occurred for flaccid but not for rigid paralysis. Kavanagh *et al.*[46] examined the expression patterns of signaling molecules implicated in regulating joint formation, GDF-5 and fibroblast growth factor (FGF)-2 and 4, in the presumptive joint regions of control and immobilized chick embryonic hindlimbs. The authors found that joint line FGF-2 expression was diminished in immobilized limbs, while FGF-4 and GDF-5 expression

patterns were unaffected by immobilization, and conclude that FGF-2 has a direct mechano-dependant role in the cavitation process.

Additional effects of immobilization

Immobilization has been used, not just for examining joint formation as described above, but for many aspects of skeletal development. Immobilization is effected in the avian embryo by cutting a "window" in the eggshell, dropping a quantity of a neuromuscular blocking agent such as decamethonium bromide[44, 47, 48] daily onto the chorioallantoic membrane, and sealing up the window with tape after each administration of the drug. Immobilization has also been effected by injecting botulinum toxin directly into the chorioallantoic vein[49]. Hosseini and Hogg[47, 48] demonstrated that immobilization has a significant effect on skeletal growth and development. They found that the lengths of many bones were reduced by immobilization, and slender bones were prone to distortion. Hall *et al.*[50] discovered that skeletal elements are differentially affected by the lack of muscle contractions, with only 27% of clavicular growth (by mass) but 77% of mandibular growth occurring in paralysed embryos. Mikic *et al.*[44] found that the menisci and sesamoids of the joints were absent for late stage immobilized chick embryos. In another study, Mikic *et al.*[51] found that the cartilage from immobilized embryos had a lower glycosaminoglycan (GAG) content, and was mechanically weaker than cartilage from control embryos. Germiller and Goldstein[52] found that immobilization led to a reduction in chondrocyte proliferation in the avian embryonic growth plate. Two methods have been used to examine the effect of mechanical forces on bone development in the embryonic rat, namely immobilization[53] and induced oligohydramnios[54]. Rodriguez et al.[53] studied the effects of fetal immobilization administering D-Tubocurarine subcutaneously to rat fetuses *in utero* from day 17 of gestation to term. The authors found that the femora of experimental fetuses exhibited alterations in shape and transverse diaphyseal growth, with

a decrease in the total cross-sectional area and in the thickness of the periosteum, and a rounder femoral cross-section in comparison to controls. Palacios et al.[54] induced oligohydramnios (a condition where motion of the fetus is restricted due to reduced amniotic fluid) by daily extraction of amniotic fluid from rat fetuses from day 17 to term. While joint development was affected, with multiple articular contractures, no alterations in femoral shape or transverse growth of the metaphysis and diaphysis were noted in the experimental fetuses, leading the authors to conclude that the main mechanical factor affecting embryonic bone development is muscular elasticity, while motion is important for fetal joint development.

Other surgical techniques can also be used to induce immobilization. Wong et al.[55] produced hindlimb muscle atrophy in the embryonic chick by excising the lumbrosacral portion of the neural tube. Rudiments from immobilized embryos were significantly shorter than controls, with greater flaring at the ends, smaller chondroepiphyses, and irregular and flattened articular surfaces. The bones from denervated embryos also had decreased mechanical strength. Therefore, a large body of evidence suggests that mechanical forces generated by muscle contractions play a role in embryonic skeletal morphogenesis.

MECHANICAL PROPERTIES OF EMBRYONIC TISSUES

Embryonic tissues have not yet been extensively characterized with respect to their mechanical properties, probably mainly due to the difficulties associated with testing very small specimens. Tanck et al.[56] examined the effect of mineralization on the mechanical properties of embryonic mouse rib rudiments using four-point bending tests in combination with FE analysis, and found an increase of two orders of magnitude during endochondral ossification. Mikic et al.[51] performed stress-relaxation mechanical tests on cores from control and immobilized embryonic chick cartilage, and found reduced values for both the

instantaneous and relaxed moduli in the cartilage from the immobilized embryos. Williamson *et al.*[57] performed static and dynamic compression tests on bovine articular cartilage, and found the confined compression modulus of embryonic cartilage increased 180% in the calf and adult. This group also tested the free swelling compressive moduli for the surface and middle layers of the articular cartilage and found a 2-2.5 fold increase in modulus between the fetal and newborn stages of development. Brown and Singerman[58] performed mechanical tests of cylindrical specimens from the chondroepiphysis of human still born femoral heads in order to calculate the equilibrium modulus. These values are summarized in Table 1.

The behavior of embryonic muscle becomes an important consideration for those working on computer models of embryonic limb morphogenesis. For such models, information on the timing and extent of the forces generated by embryonic muscle contractions is critical. Landmesser and Morris[59] describe the development of functional innervation in the hind limb of the chick embryo between stages HH25 and 43. The authors found that most limb muscles or primitive muscle masses become functionally innervated at the same time at around stage 27-28, just prior to regular movement of the limbs in the chick embryo. The authors also provide isometric twitch tension data for stage 33 chick muscles from which force per unit area values can be deduced. Reiser and Stokes[60] describe the development of contractile properties of avian embryonic skeletal muscle in terms of twitch and tetanic responses, including force per unit mass values. The authors found that in the last week *in ovo*, the normalized twitch and tetanic forces of the posterior latissmus dorsi muscle, normalised to the mass, increased 3- and 12- fold respectively; from 0.53 mN/mg to 2.10 mN/mg for the twitch response and from 0.76 mN/mg to 9.15 mN/mg for the tetanic response. A decrease in the time to peak twitch force and time to one-half relaxation of the twitch response also took place over this time period. Kardon[42] provides a thorough account of muscle and tendon morphogenesis in the

embryonic chick limb, and describes the close association of the development of these two tissues types.

COMPUTATIONAL MODELS

Computational mechanobiology problems may be classified into two types[61]. The first kind is a single time-point analysis where biophysical stimuli are computed and used to predict tissue differentiation and remodelling when the forces are known. The second kind - we may call it simulation modeling - takes the initial condition and simulates a process of adaptation and differentiation over time. The most commonly used method for computing biophysical stimuli is FE analysis, a numerical method where computers find approximate solutions to large sets of equations[62].

Single Time-Point Analyses

Carter and co-workers have used computational mechanobiology to examine many aspects of skeletal development such as the influence of stresses on embryonic chondrogenesis and osteogenesis[63], alteration of ossification in culture[64], the evolution of long bone epiphyses[65] and sesamoid bone formation[66].

In 1987 Carter *et al.*[67] hypothesized, following Pauwels[68], that mechanical stresses influence chondroosseous biology through a combination of intermittently applied shear stresses (or strain energy) and hydrostatic (dilatational) pressure in an FE model. A plane-strain 2-D FE model of the human femur was created for 5 time-points, 3 embryonic stages and 2 post-natal stages. The same shape and loads were used throughout and the material properties assigned were changed to reflect the pattern of ossification. Although a much simplified model, it gives insightful results. High strain energy density values are predicted at the midshaft region in the all cartilage model but, once ossification has begun, strain energy density is highest immediately ahead of the front, peaking at the periosteal surface. In later stages, the region of high strain energy shifts to the centre of the chondroepiphysis,

where the secondary ossification centre appears. Biaxial compressive stresses are predicted near the joint surface and therefore the authors propose that this stimulus inhibits ossification, enabling maintenance of articular cartilage.

Wong and Carter[64], performed an analysis of the Klein-Nulend *et al.* embryonic mouse metatarsal organ culture experiment[25], where intermittent hydrostatic pressure was found to lead to increased calcification in long bone rudiments *in vitro*. An osteogenic index[69], a combination of the influence of the tissue shear and hydrostatic stresses, was used to predict ossification patterns. The index is given by

$$I = \sum_{i=1}^{c} n_i (S_i + kD_i)$$

where n_i = number of load cycles of load case i, S_i = cyclic octahedral stress, D_i = dilatational (hydrostatic) stress, k= empirical constant, and c = total number of load cases. The authors hypothesized that, once calcification had begun, externally applied hydrostatic pressure produced local shear stresses at the mineralization front, which may have led to increased calcification rates. When a model of the same experiment was created by Tanck *et al.*[70], with poroelastic (fluid and solid phase) instead of single-phase elastic properties, the hypothesis of Wong and Carter[64] could not be confirmed. Results from this axi-symmetric poroelastic FE model indicated that hydrostatic pressure, rather than distortional strain were likely to have enhanced the mineralization process.

Tanck *et al.*[26] also performed a 3-D poroelastic FE analysis in order to explore the results of another organ culture experiment where a curved mineralization front was found in explanted embryonic mouse metatarsal rudiments (as illustrated in Figure 4). The results of the model indicated that fluid pressure was approximately the same at the center as at the periphery during flexion and extension contractions, and since the rate of mineralization was higher at the centre, pressure was unlikely to be involved in regulation

of growth of the mineralization front. The authors concluded that the distributions of distortional strain were compatible with the difference in mineralization geometry.

In a study examining the evolution of epiphyses, Carter *et al.*[65] constructed two different FE models of the embryonic chondroepiphysis to represent (1) a basal tetrapod and (2) a mammal. Basal tetrapod refers to the first tetrapods which emerged from the water during the Devonian and Carboniferous periods, whose long bones are thought to have developed like those of extant crocodiles, turtles and birds. In the basal tetrapod bone, periosteal ossification advanced faster than endochondral ossification, leaving cartilage in the form of cartilage cones which disappeared as development progressed. The two long bone models analyzed involved a model with cartilage cones and little cancellous bone (basal tetrapods, crocodiles, turtles and birds), and a model with well-ossified cancellous bone (mammals). In the basal tetrapod FE model, the cancellous bone of the epiphysis is not as dense as the mammalian cancellous bone, which leads to a lower osteogenic index in the chondroepiphysis of the basal tetrapod. The authors suggest that this may be why secondary ossification centers do not always form in the extant forms of the basal tetrapods (e.g., in birds, as illustrated in Fig. 3).

Sarin and Carter[66] used FE analysis to investigate endochondral ossification of sesamoid bones such as the patella. A 2-D linear elastic model of a sesamoid cartilage embedded within a fibrous tendon that wraps around a bone prominence was performed. The authors found that high contact pressures inhibit ossification and promote the maintenance of an articular cartilage layer, and high octahedral pressures predict regions favorable for the onset of endochondral ossification as found in sesamoids *in vivo*.

Nowlan *et al.*[71, 72] presented a model of avian embryonic skeletal development. This model of the embryonic avian long bone rudiment includes realistic morphologies and muscle forces based on the imaging of actual specimens at a range of developmental stages around the time of onset of ossification. The model can be used to examine the changing

stresses, strains, pressures and fluid velocities at each stage examined. The novelty of this approach lies in using the scanning technique Optical Projection Tomography[73] (OPT) to obtain 3-D images of embryonic tissues stained either colormetrically or fluorescently to highlight different tissues. The 3-D images obtained can be converted to a format suitable for FE analysis, as shown in Figure 6.

Immunohistochemistry with an anti-myosin antibody is used to obtain muscle morphology, and muscle forces are calculated from these data. A poroelastic analysis is performed in Abaqus[®], with material properties from studies by Tanck[26, 56]. In the model, one loading cycle consists of two muscle contractions, flexion followed by extension, as performed by Tanck *et al.*[26]. While patterns of hydrostatic pressure did not change over the stages examined, concentrations of fluid flow and shear strain change in pattern and are found at the periosteal surface of the rudiment at the location where ossification will take place some hours later, suggesting that shear strain and/or fluid velocity, rather than fluid pressure, may initiate cartilage hypertrophy and subsequent periosteal ossification in the avian embryonic long bone. The model addresses several of the limitations of previous computational models of embryonic rudiments, such as geometric complexity, and provides a useful tool with which to test a range of hypotheses, such as combined strain/fluid flow control of stem cell differentiation[74]. In order to test this hypothesis, a stimulus *S* is used to combine octahedral shear strain and fluid flow, where S is given by

$$S = \frac{\tau_{oct}}{a} + \frac{\vec{v}}{b}$$

as shown in Figure 8.

Simulation Models

Simulation type models have been created to model growth and endochondral ossification[75, 76], joint development[77] and growth front morphology in developmental dysplasia of the hip[5]. Stevens *et al.*[75] created a model of long bone development incorporating biological and mechanobiological influences[78] on endochondral growth and ossification. A time-dependant linear elastic FE model was used to 'grow' the rudiment according to a bone-remodeling algorithm dependant on a calculated cartilage maturation rate. Despite some simplifications such as isometric scaling and constant joint pressures throughout, the results of the simulation predicted events such as a secondary ossification center, and formation of the growth plate and articular cartilage, as shown in Figure 8.

Heegaard *et al.*[77] simulated morphogenesis of a human interphalangeal joint between days 55 and 70 of fetal life. When using a growth rate based on biological and mechanobiological influences, they found that more congruent articular joint surfaces were formed, whereas if only a baseline biological rate was used the epiphyses would increase in size but remain incongruent. The authors conclude that mechanics are critical to normal joint morphogenesis.

Shefelbine and Carter[5] described a 3-D model of a third trimester human femur in order to investigate if alterations in hip joint reaction forces could explain abnormal growth front morphology and bony deformities in patients with developmental dysplasia of the hip (DDH). A growth rate based on biological and mechanobiological contributions was used to predict growth front morphology for normal and a range of DDH load histories (greater hip force angles). The octahedral and hydrostatic stress patterns found by the model, as shown in Figure 9, predicted that abnormal loads would lead to altered growth front morphology resulting in coxa valga (large neck-shaft) in DDH. The interaction between the perichondrium and the PTHrP-Ihh control loop is simulated by van Donkelaar et al.[76], and the results indicate that the location of the perichondrium determines the pattern of early mineralization in a cartilaginous anlage.

CONCLUSION

Chick and mouse have been the most commonly used animals for experiments in skeletal mechanobiology. The advantages of the chick embryonic model are that it can be manipulated and altered in a way that is impossible in the mammalian embryo, but, as described in the introduction, chick long bone osteogenesis is significantly different from mammalian osteogenesis. Nonetheless, we can use the chick system to understand the mechanical environment in the early cartilaginous rudiments and the stimuli that might trigger subsequent events. The chick embryo can also be used to study bone collar formation and secondary ossification centers, while we must look to the mouse or rat to investigate the primary and secondary ossification processes. We speculate that mechanical forces due to muscle contractions up- or down-regulate certain mechanosensitive genes, such as Ihh or BMP-2/4, that influence ossification events in the embryonic skeleton. By comparing gene expression patterns between control and immobilized embryos (altered mechanical environment), and between chick and mouse (different ossification sequences), one or more mechanotransduction pathways could be identified to provide a causal link between mechanical forces and developmental change.

With the advent of new imaging techniques such as OPT, computational modeling in development could enter a new phase. We envisage computer simulation of limb development and growth accounting for factors such as genetic events, mechanical properties and individual variability[79]. Indeed, such computational simulations could be useful not only for scientific investigation but also, ultimately, as clinical tools for treatment of neuromuscular disorders such as spinal muscular atrophy.

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Figure 1. *Radiograph of a newborn infant with spinal muscular atrophy, showing complete fracture of the right humerus and left ulna and radius. The left humerus has a fracture of the cortical bone only (arrow) (from Rodríguez et al.[3]).*

Figure 2. *Longitudinal sections of human embryonic femora at (A) 8, (B) 11 and (C) 35 weeks of gestation. At 8 weeks, the femur is 4.2 mm long, and the periosteal bone collar has formed (between arrows). At 11 weeks, the femur is 16.7 mm long. Endochondral ossification, trabeculae and forming marrow cavity are present at the mid-diaphysis. At 35 weeks (length=70 mm), mineralization fronts have advanced to form epiphyses at the ends of the diaphyses (adapted from Gardner & Gray[10])*

Figure 3. *Mammalian and avian long bone development begins with a cartilaginous template (A). Next, the chondrocytes in the mid-diaphysis undergo hypertrophy (B). In the mammal (C-G), the cartilage is first invaded by capillaries (C) before the periosteal bone collar forms (D). Blood vessels penetrate to the middle of the rudiment, which undergoes endochondral ossification (E). Growth fronts progress and some bone is resorbed to form the marrow cavity (F). Secondary ossification centers form in most mammalian long bones (G). In the bird (H-K), periosteal ossification (H) occurs before vascularization (I). As the collar grows, cartilage is resorbed to form the marrow cavity (J). Only in some long bones in the bird will a secondary ossification centre form after hatching (K) (see text for references).*

Figure 4. *(A) Difference in length of the mineralized zone between the centre and periphery pf the metatarsal diaphysis (E17= mineralized metatarsal at 15 days of gestation, E15+5= metatarsal at 15 days of gestation with 5 days of culture, E17+3= metatarsal at 17 days of gestation with 3 days of culture, E18= metatarsal at 18 days of gestation). *p<0.05, compared with E17 and E18. Error bars represent the SD. (B) Representative pictures of the metatarsals from the four groups, from left to right: E17, E15+5, E17+3, E18 (from Tanck et al.[26]).*

Figure 5. *Explanted knee joints of 7-day old chick embryos, cultivated for 6 days; in (A) the explant was not moved and resulted in cartilaginous fusion of the femur and tibia; in (B) the joint was moved 5 times a day and articular surfaces are well developed (adapted from Lelkes[30])*

Figure 6. *A 3-D image (VTK format) from an OPT scan is processed in Rhino® and Cubit® before FE analysis*

Figure 7. *Stimulus patterns mid-extension contraction, ventral aspect of the tibiotarsus rudiment at 6, 7 and 8 days of incubation (HH30, HH32 and HH34).*

Figure 8. *Simulation results predict the formation of a secondary ossification centre, growth plate and articular cartilage (from Stevens* et al. [75])

Figure 9. *Minimum hydrostatic stress and maximum octahedral stress patterns for normal and developmental dysplasia of the hip (DDH) loading histories (adapted from Shefelbine and Carter[5])*

Authors	Animal	Embryonic Age	Embryonic Tissue Type	Parameter Measured	Value
Tanck et al.[56]	Mouse	16 and 17 embryonic days	Cartilage	Young's Modulus	1.11 ± 0.62 MPa
			Calcified Cartilage	Young's Modulus	117 ± 62 MPa
Mikic et al.[51]	Chick (Control)	Day 14 of incubation	Cartilage	Instantaneous Modulus	0.25 MPa
				Equilibrium Modulus	0.08 MPa
	Chick (Immobilized)		Cartilage	Instantaneous Modulus	0.125 MPa
				Equilibrium Modulus	0.058 MPa
Williamson et al.[57]	Bovine	Mid-third trimester (238.2 days)	Articular Cartilage	Confined Compressive Mod.	0.11 ± 0.03 MPa
			Articular Cartilage (surface layers)	Free swelling compressive Mod.	0.106 ± 0.007 MPa
			Articular Cartilage (middle layers)	Free swelling compressive Mod.	0.153 ± 0.003 MPa
Brown & Singerman[58]	Human	Full term (9 months)	Chondroepiphysis of femoral head	Equilibrium Modulus	0.699 MPa
				Permeability	2.5×10^{-15} m^{4}Ns^{-1}

Table 1. *Mechanical properties of mouse, chick, bovine and human embryonic cartilaginous*

tissue