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Characterising the effects of *in vitro* mechanical stimulation on morphogenesis of developing limb explants

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ABSTRACT

Mechanical forces due to fetal movements play an important role in joint shape morphogenesis, and abnormalities of the joints relating to abnormal fetal movements can have long-term health implications. While mechanical stimulation during development has been shown to be important for joint shape, the relationship between the quantity of mechanical stimulation and the growth and shape change of developing cartilage has not been quantified. In this study, we culture embryonic chick limb explants *in vitro* in order to reveal how the magnitude of applied movement affects key aspects of the developing joint shape. We hypothesise that joint shape is affected by movement magnitude in a dose-dependent manner, and that a movement regime most representative of physiological fetal movements will promote characteristics of normal shape development. Chick hindlimbs harvested at seven days of incubation were cultured for six days, under either static conditions or one of three different dynamic movement regimes, then assessed for joint shape, cell survival and proliferation. We demonstrate that a physiological magnitude of movement *in vitro* promotes the most normal progression of joint morphogenesis, and that either under-stimulation or over-stimulation has detrimental effects. Providing insight into the optimal level of mechanical stimulation for cartilage growth and morphogenesis is pertinent to gaining a greater understanding of the etiology of conditions such as developmental dysplasia of the hip, and is also valuable for cartilage tissue engineering.

1. Introduction

Each type of synovial joint has a highly specialised shape, and alterations or abnormalities in the development of these shapes can compromise their functionality. Reduced fetal movements are implicated in musculoskeletal conditions of impaired joint shape development, such as development dysplasia of the hip and arthrogryposis (reviewed in Nowlan (2015)). However, it is unclear how the quantity, timing and type of mechanical stimulation due to fetal movements influence joint shape morphogenesis. This question is relevant to lifelong musculoskeletal health, as developmental joint abnormalities can affect the joint's range of motion and the transmission of mechanical loads, increasing the risk of degenerative joint diseases such as osteoarthritis later in life (Sandell, 2012). Furthermore, a better understanding of how mechanical stimulation directs or determines cartilage growth during prenatal development is highly relevant to cartilage tissue engineering, in which the aim is to recapitulate the developmental processes occurring in fetal rudiments.

Previous studies have explored the influence of mechanical stimuli on skeletal development using animal models of reduced, absent or abnormal fetal movements (reviewed in Nowlan et al. (2010b)). While many of these studies focused on cavitation or ossification rather than joint morphogenesis, we do have some understanding of the effects of immobility on joint shape development. In immobilised chicks embryos, articular joints are often fused across the joint site, and normal interlocking joint shapes are lost (Drachman and Sokoloff, 1966; Hall and Herring, 1990; Hosseini and Hogg, 1991; Nowlan et al., 2014; Osborne et al., 2002; Roddy et al., 2011b). Paralysis of zebrafish embryos leads to alterations in jaw joint shape and inhibition of normal joint function (Brunt et al., 2015). Failure to produce joint cavities and abnormal joint shapes have also been observed in 'muscle-less' mice embryos (Kahn et al., 2009; Nowlan et al., 2010a) along with signs of irregular joint shape development in 'reduced muscle' mice embryos (Kahn et al., 2009). Early studies used *in vitro* culture methods to investigate the role of movement on joint development. Explants from four day old embryos failed to form a complete knee (stifle) joint after six days of static culture *in vitro*, using the "watch glass" technique (Fell and Canti, 1934). However, when six or seven day old embryonic chick knee explants were

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cultured for six days by Lelkes (1958), manual manipulation of the explants led to cavitation and development of articular surfaces between the femur and tibia (or tibiotarsus). Since these pioneering papers were published, *in vitro* culture methods have improved dramatically. Modern bioreactors enable repeatable cultivation of tissue and application of controlled mechanical stimulation in ways that are not possible *in vivo* (Cohen et al., 2005; Pörtner et al., 2005). *In vitro* culture of embryonic chick hindlimb elements has been shown to be a versatile model for studying skeletal development (Smith et al., 2013), and a bioreactor system has been used to apply cyclic hydrostatic pressure to promote bone growth and mineralisation in embryonic chick femurs (Henstock et al., 2013). A recent feasibility study showed the whole chick hindlimb could be cultured whilst applying flexion and extension movements to the knee joint (Rodriguez and Munasinghe, 2016). However, the quantitative relationship between mechanical stimulation and joint morphogenesis has not been described, a deficit that is addressed in this current study.

In this study, a novel 3D explant culture system is used to investigate the development of the embryonic chick knee joint under a range of flexion movement regimes, with the aim of characterising the relationship between the magnitude of applied movements and key aspects of fetal joint morphogenesis. It was hypothesised that joint shape development would be affected by movement magnitude in a dose-dependent manner, and that the most physiological movement regime would lead to a joint with the most normal progression of shape morphogenesis.

2. Methods

2.1. Characterisation of physiological knee morphology

To evaluate the progression of joint shape development in cultured explants, we first analysed the morphology of the knee joint over 7 to 9 days of incubation, a period of dramatic shape change for the joint. Limbs were processed for 3D shape and size analysis as described below.

2.2. Preparation of explants for culture

Fertilised white DeKalb eggs (Henry Stewart & Co, UK) were incubated at 37 °C under humidified conditions for seven days. Hindlimbs were harvested, the digits removed, and the soft tissues surrounding the rudiments removed as described by Henstock et al. (2013). Preliminary experiments demonstrated that this step of soft tissue removal increased the duration of time that the explant could be viably maintained *in vitro* (data not shown).

2.3. Explant culture setup

Rectangular pieces ($35 \times 20 \times 15$ mm^3) of polyurethane foam (Sydney Heath & Son, UK) were used to support the hindlimb explants during culture. The foam support was cut to create a step running horizontally along the top surface (Fig. 1A). Each hindlimb was positioned, medial side down, onto the lower level and oriented with the distal end nearest the step (Fig. 1B). Six specimens were placed on each support (Fig. 1B). Once positioned, each explant was pinned to the support using a 27G needle through the superior part of the pelvis to secure the limb. The foam supports were transferred into a uniaxial compression bioreactor (Ebers TC-3, Spain) and filled with basal culture media (alpha – minimum essential media (α-MEM GlutaMAX, Gibco) supplemented with 1% pen/strep, 1% Amphotericin B and 100 μM Ascorbic Acid (Sigma, UK)). The explants were maintained at an air–liquid interface, and the culture medium was replenished every 24 h.

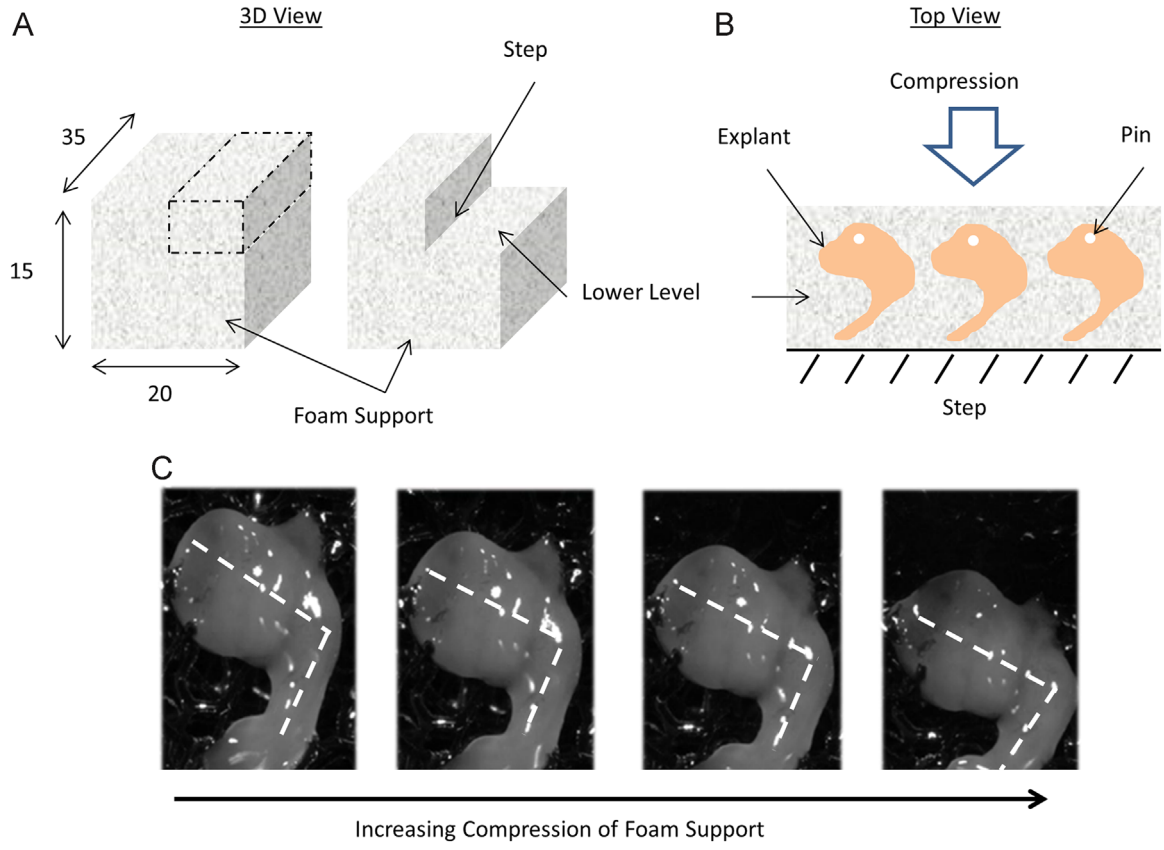

Fig. 1. Dynamic explant culture setup. A) A polyurethane foam support was used to support the hindlimb explants during culture. The rectangular piece of foam was cut to create a step along the horizontal axis. Measurements are in mm. B) Explants were positioned onto the lower level of the foam and pinned into place. Explants were orientated medial side down with the hip joint furthest away from the step. C) Uniaxial compression of the foam support caused the hindlimb to bend at the knee joint mimicking a flexion motion of the knee joint.