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Differential effects of altered patterns of movement and strain on joint cell behaviour and skeletal morphogenesis

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SUMMARY

Objective: There is increasing evidence that joint shape is a potent predictor of osteoarthritis (OA) risk; yet the cellular events underpinning joint morphogenesis remain unclear. We sought to develop a genetically tractable animal model to study the events controlling joint morphogenesis.
Design: Zebrafish larvae were subjected to periods of flaccid paralysis, rigid paralysis or hyperactivity. Immunohistochemistry and transgenic reporters were used to monitor changes to muscle and cartilage. Finite Element Models were generated to investigate the mechanical conditions of rigid paralysis. Principal component analysis was used to test variations in skeletal morphology and metrics for shape, orientation and size were applied to describe cell behaviour.
Results: We show that flaccid and rigid paralysis and hypermobility affect cartilage element and joint shape. We describe differences between flaccid and rigid paralysis in regions showing high principal strain upon muscle contraction. We identify that altered shape and high strain occur in regions of cell differentiation and we show statistically significant changes to cell maturity occur in these regions in paralysed and hypermobile zebrafish.
Conclusion: While flaccid and rigid paralysis and hypermobility affect skeletal morphogenesis they do so in subtly different ways. We show that some cartilage regions are unaffected in conditions such as rigid paralysis where static force is applied, whereas joint morphogenesis is perturbed by both flaccid and rigid paralysis; suggesting that joints require dynamic movement for accurate morphogenesis. A better understanding of how biomechanics impacts skeletal cell behaviour will improve our understanding of how foetal mechanics shape the developing joint.

Introduction

We now understand that the mechanical environment experienced during early development is important for normal skeletal development. There are multiple conditions for which abnormal or reduced movement are causal; including developmental dysplasia of the hip (DDH), which affects 1.3 per 1000 births[1,2], arthrogryposis which affects around 1:4000 births[3] and fetal akinesia deformation sequence (FADS) which affects 1:15,000 births[4,5]. Additionally, there is evidence that early changes to joint shape lead to osteoarthritis (OA) later in life[6]. This can arise if conditions such as DDH are uncorrected[7], but also subtle changes to hip shape have been identified as conferring increased risk of OA[8,9]. Despite the clinical significance of joint shape, relatively little is known about the underlying cellular events underpinning joint morphogenesis[10,11].

Many studies have investigated the effect of temporal paralysis on joint formation. The majority have been undertaken in developing chick and mouse limbs and have shown that paralysis caused flattening of articular surfaces and a failure of joint cavitation,

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resulting in fusion of opposing elements[12–15]. By contrast, few studies have focused on the role of biomechanics in craniofacial development, though craniofacial morphogenesis is also affected by paralysis, with different joints differentially affected in chicks[13,16,17]. It is less clear what the effect of more sustained hyperactivity of the system will be and whether this would be beneficial to the skeletal system; for example, in chick, the effects of hypermobility have been described to increase joint cavity size[18].

Previously it was believed that joint morphology developed after cavitation, however, recent studies of chick knee and hip joints have revealed that morphogenesis precedes cavitation, with most anatomical features present prior to element separation[19,20], lineage tracing in mouse also reveals morphogenesis prior to separation[21].

We still know relatively little about the cellular events that underpin morphogenesis; though recent work has started to address this question. In chick knee development, patterns of mechanical strain co-localise with regions of increased cellular proliferation, giving clues that *in vivo* cellular behaviour is altered mechanically[22]. In zebrafish, movement is required for normal chondrocyte intercalation[23] and correct cell orientation at the joint[24]. Recently, there has been increased focus on identifying putative mechanosensitive genes that could couple mechanical forces to downstream morphological responses[14,25,26]. Zebrafish, with their many transgenic lines marking various cell types of the musculoskeletal system[27] raise the prospect of using imaging to help unravel the cellular dynamics that underpin skeletal morphogenesis. We wanted to compare the effects of rigid paralysis and hyperactivity with flaccid paralysis and observe their impact on jaw joint morphology.

Materials and methods

Zebrafish husbandry

Zebrafish were housed as previously described[28]. Animal experiments were ethically approved by the local ethics committee and by the Home Office.

Pharmacological treatment

Fish were treated from 3 days post fertilisation (dpf) to 5 dpf, with drugs replaced twice daily diluted in Danieau buffer in petri dishes[28]. Flaccid paralysis anaesthetic MS222 (Tricaine methanesulfonate), (Sigma) was used at 0.1 mg/ml. Decamethonium bromide (DMB) induces rigid paralysis and has been used to induce paralysis in chicks in ovo leading to alterations to joint patterning[12–14]. DMB was used at 8 mg/ml diluted into Danieau buffer. 4-amino-pyridine (4-AP), a potassium channel antagonist can induce hyperactivity in chick foetuses[29]. 4AP was tested at concentrations from 0.05 mM to 1.2 mM and 0.5 mM was selected for further analysis.

Tracking of fish swim motility

Swim motility was measured by tracking individual control or 4AP-treated fish from movies. Tracking was performed using a manual tracking ImageJ plugin[30], which when calibrated for pixel size and time interval between frames allows quantification of distance travelled and velocity (Sup. Vid. 1). Measurements were made on 10 fish per 4-AP dose per time period of drug application.

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.joca.2016.06.015

Recording frequency of jaw movement

Zebrafish were anaesthetised with MS222 and mounted laterally onto coverslips in 1% agarose. The agarose surrounding the head was removed and Danieau buffer flushed over the coverslip until jaw movements resumed. The number of mouth movements per minute was recorded from four fish per timepoint with 3 measurements taken per fish and mean values used (Sup. Vid. 2). 2-tailed students *t*-tests were used to compare control with 4AP-treated larvae and siblings with *vhl* mutants.

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Measurement of jaw displacement

High-speed movies were made of jaw movements in wild type and *vhl* mutants; frames corresponding to maximum jaw displacements were selected, imported into ImageJ[31] and measurements taken on the distance between the tip of the Meckel's cartilage (MC) in the lower jaw and upper jaw in μm (Sup. Fig. 1). Two-tailed Student *t* test was used to compare average displacements from *vhl* mutants to controls.

Zebrafish lines

All transgenic and mutant lines have been previously described: Col2a1:mcherry[32,33]; myod^{fh261}[34]; VHL *vhl*^{hu2117}[35]; *Tg(-4725sox10:GFP)*^{ba4}[36]; symhc:GFP[37]. *Vhl* mutants, in which Hif2a is stabilised, display a hypoxic response despite being well oxygenated[35,38].

Wholemount immunohistochemistry

Immunohistochemistry was performed as previously described[24]. Larvae were fixed in 4% PFA and stored in 100% MeOH, rehydrated into PBS with 0.1% Tween (PBSTw), permeabilised using 15 μg/ml proteinase K, washed and blocked in PBS+ 5% horse serum for at least 2 h. The larvae were incubated with anti-myosin A4.1025 mouse IgG [1:200 dilution; Developmental Studies Hybridoma Bank (DSHB)] or rabbit anti-col2 IgG (1:500 dilution, Abcam) in blocking solution overnight at 4°C and washed a minimum of four times in 1×PBSTw. Larvae were incubated with secondary antibodies (Dylite 488 goat anti-mouse IgG and Dylite 550 goat anti-rabbit IgG, Molecular Probes, 1:500 dilution) then washed extensively in 1×PBSTw prior to visualisation. Controls were exposed to only secondary antibodies.

Analysis of shape variation

Changes to MC shape caused by flaccid paralysis (MS222, *Myod*), rigid paralysis and hyperactivity were quantified using two-dimensional (2D) geometric morphometrics. 2D MC outlines [Sup. Fig. 2(A)] of 5dpf controls ($n = 15$), *vhl* mutants ($n = 4$), *Myod* mutants ($n = 8$) were prepared (Adobe Illustrator) and compared with outlines from larvae treated from 3-5dpf with MS222 ($n = 18$) and DMB ($n = 4$). The outlines were converted into 200 XY coordinates with a common origin located at the anterior tip using TpsDig 2.25[39] [Sup. Fig. 2(B)]. Coordinates were converted to sine and cosine components using Hangle Fourier transformations[40] and superimposed using Procrustes superimposition. To assess shape variation qualitatively, the data were subjected to a between groups principal components analysis (PCA) and one-way non-parametric MANOVA. The analyses were performed using Paleontological statistics software (Past 2.17)[41].