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Skeletal muscle contractions are critical for normal skeletal growth and morphogenesis, but it is unclear how the detrimental effects of absent muscle on the bones and joints change over time. Joint shape and cavitation, and rudiment length and mineralisation were assessed in multiple rudiments at two developmental stages (Theiler Stage (TS)24 and TS27) in the splotch-delayed "muscleless limb" mouse model and littermate controls. Chondrocyte morphology was quantified in 3D in the distal humerus at the same stages. As development progressed, the effects of absent muscle on all parameters except for cavitation become less severe. All major joints in muscleless limbs were abnormally shaped at TS24, while, by TS27, most muscleless limb joint shapes were normal, or nearly normal. In contrast, any joints which were fused at TS24 did not cavitate by TS27. At TS24, chondrocytes in the distal humerus were significantly smaller in the muscleless limbs than in controls, while by TS27, chondrocyte volume was similar between the two groups, offering a cell-level mechanism for the partial recovery in the shape of muscleless limbs. Mineralisation showed the most pronounced changes over gestation. At TS24, all muscleless rudiments studied had less mineralisation than the controls, while at TS27, muscleless limb rudiments had mineralisation extents equivalent to controls. We conclude that the effects of absent muscle on prenatal murine skeletogenesis reduce in severity over gestation. Understanding how mammalian bones and joints continue to develop in an environment with abnormal fetal movements provides insights into conditions including hip dysplasia and arthrogryposis.
Prenatal murine skeletogenesis partially recovers from absent skeletal muscle as development progresses

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Running title: Recovery of skeletogenesis despite absent muscle

Abstract

Skeletal muscle contractions are critical for normal skeletal growth and morphogenesis, but it is unclear how the detrimental effects of absent muscle on the bones and joints change over time. Joint shape and cavitation, and rudiment length and mineralisation were assessed in multiple rudiments at two developmental stages (Theiler Stage (TS)24 and TS27) in the splotch-delayed “muscleless limb” mouse model and littermate controls. Chondrocyte morphology was quantified in 3D in the distal humerus at the same stages. As development progressed, the effects of absent muscle on all parameters except for cavitation become less severe. All major joints in muscleless limbs were abnormally shaped at TS24, while, by TS27, most muscleless limb joint shapes were normal, or nearly normal. In contrast, any joints which were fused at TS24 did not cavitate by TS27. At TS24, chondrocytes in the distal humerus were significantly smaller in the muscleless limbs than in controls, while by TS27, chondrocyte volume was similar between the two groups, offering a cell-level mechanism for the partial recovery in the shape of muscleless limbs. Mineralisation showed the most pronounced changes over gestation. At TS24, all muscleless rudiments studied had less mineralisation than the controls, while at TS27, muscleless limb rudiments had mineralisation extents equivalent to controls. We conclude that the effects of absent muscle on prenatal murine skeletogenesis reduce in severity over gestation. Understanding how mammalian bones and joints continue to develop in an environment with abnormal fetal movements provides insights into conditions including hip dysplasia and arthrogryposis.

Keywords

Prenatal skeletal development, fetal movements, developmental biomechanics, cartilage, bone, morphogenesis, ossification, mouse.

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Introduction

Fetal movements play an important role in prenatal skeletal development (Nowlan, 2015). When skeletal muscle in animal models is absent, reduced in volume or non-contractile, skeletal rudiments tend to be shorter, with decreased mineralisation and malformed joints (Bridglal et al., 2021; Brunt et al., 2016; Kahn et al., 2009; Khatib et al., 2021; Nowlan et al., 2008; Nowlan et al., 2010; Nowlan et al., 2014; Pierantoni et al., 2021; Roddy et al., 2011; Sotiriou et al., 2019). Vertebral segmentation, vertebral shape and intervertebral disc formation are also dependent on muscle forces (Levillain et al., 2019; Levillain et al., 2021; Rolfe et al., 2017). Not all bones are affected equally by compromised skeletal muscles, with the bones and joints of the forelimb being more severely affected than those of the hindlimb (Kahn et al., 2009; Nowlan et al., 2010; Sotiriou et al., 2019). Unequal stimulation of the muscleless limbs from passive movements has been proposed as a possible mechanism underlying the differential effects of absent muscle on skeletal development (Nowlan et al., 2012). While it is clear that skeletal muscle contractions are critical for normal growth and morphogenesis of most bones in the limbs and spine, the effects of abnormal skeletal muscle on mammalian skeletal development over time in utero have not been characterised in detail.

A small number of prior studies have investigated the temporal effects of mechanical forces due to skeletal muscle contractions over development. Very early stages of skeletal development occur independently of skeletal muscle contractions, including interzone formation (Kahn et al., 2009; Mikic et al., 2000), joint morphogenesis prior to cavitation (Nowlan and Sharpe, 2014) and notochord involution (Levillain et al., 2021). The authors recently showed that the time window between embryonic days four and seven is the most important period for muscle forces in terms of the effects on hip joint development in the chick embryo (Bridglal et al., 2021), but that the effects of immobilisation do not become pronounced until day eight and later (Nowlan et al., 2014). Also in the chick embryo, the authors showed that a single day of immobilisation at embryonic days three or four had pronounced, lasting effects on spinal curvature, vertebral segmentation and vertebral shape, while single-day immobilisation at embryonic day five led to the most severe rib abnormalities (Levillain et al., 2019). Pollard et al. (2017) found that the area of the furcula (part of the pectoral girdle in birds) was unchanged by short period of flaccid paralysis, but significantly reduced by sustained flaccid paralysis between embryonic days 10 and 18. Drachman and Coulombre (1962) reported relatively severe effects at hatching on the skeleton after one or two days of paralysis between seven and nine days of incubation, indicating that the effects of temporary paralysis (at least in the chick) may become more severe as development progresses. Most published works with a temporal component use the chick or fish model system, and only a small number of studies using mammalian model systems have assessed the effects of absent or abnormal muscle on skeletal development over time (Kahn et al., 2009; Pierantoni et al., 2021). Kahn et al. (2009) assessed joint development in mice with absent or non-contractile skeletal muscle at multiple developmental stages (embryonic day (e)12.5, 13.5, 14.5, 16.5 and 18.5), and reported that elbow joint fusion, first detectable at e16.5 (ca. TS25) is maintained until e18.5 (equivalent to TS27). Joint shape was not quantitatively assessed. Pierantoni et al. (2021) quantified humeral bone properties including bone volume and extent at TS23, TS24 and TS27 in muscleless limb and control embryos, and report that while many mineralisation parameters were significantly different at TS24, mineralisation had caught up in the muscleless limb humeri by TS27. Temporal characterisation on the effects of absent muscle on rudiments other than the humerus are lacking, and the progression of joint shape in the absence of skeletal muscle has not been quantified. Given the known differential effects of absent or abnormal muscles on different skeletal rudiments (Kahn et al., 2009; Nowlan et al., 2010), a holistic study of the effects of absent muscle on all key aspects of the developing mammalian skeleton is warranted.
In this work, the progression of prenatal skeletal development when skeletal muscles are absent was investigated, to determine if the effects on the bones and joints remained constant, worsen, or improve over developmental time. Understanding the temporal effects of inter-uterine immobility has impact for conditions such as amyoplasia and developmental dysplasia of the hip, in which an early, often temporary, period of restricted or reduced movement can have long-lasting consequences on joint shape (Nowlan, 2015). In this research, key parameters of skeletal development were assessed, namely joint size, shape and cavitation, and rudiment length and mineralisation, for multiple rudiments and over two developmental stages; Theiler Stage (TS)24 (e15.5) and TS27 (e18.5), in the splotch-delayed “muscleless limb” mouse model and littermate controls. 3D data was obtained using optical projection tomography (OPT), followed by rigid image registration, to characterise morphology, and histology was used for assessment of cavitation. Finally, the cell-level events underlying the temporal effects of absent skeletal muscle on joint development were explored. Muscleless limbs and littermate control limbs at TS24 and TS27 were imaged using high resolution phase-contrast synchrotron X-ray tomography, and matrix proportion, chondrocyte volume, and chondrocyte orientation in the distal humerus were compared between groups.

Methods

Animal model

All animal experiments were performed in accordance with European legislation (Directive 2010/63/EU). Embryos from the Pax3SpSp Splotch delayed (Spd) mice were studied. In Pax3 mutants, muscle progenitor cells do not migrate to the limb buds and thus, the limbs are devoid of skeletal muscle (Franz et al., 1993). Homozygous mutations in Pax3 are neonatal lethal, while heterozygous embryos have no limb muscle abnormalities (Franz et al., 1993). Heterozygous adult animals were imported from The Jackson Laboratory (Maine, USA; JAX stock #000565) and interbred. Pregnant mice were humanely sacrificed using cervical dislocation and embryos were euthanised and staged according to Theiler’s Staging criteria (Theiler, 1989). Genotyping was done by PCR on DNA derived from head tissue. The PCR reaction was carried out for 30 cycles, each with a duration of 30 seconds at 94 °C, 60 °C and 74°C, using three primers. Primer sequences used were AGGGCCGAGTCAACCAGCA & CACGCGAAGCTGGCGAGAAATG for controls and AGTGCACCCTCTGGGCCCTCGGCCAGTCAACCAGGTCC & CACGCGAAGCTGGCGAGAAATG for mutants. For the organ-level morphological analyses, five muscleless and littermate control embryos at TS24 and at TS27 were analysed in detail in this study. The control embryos came from one (TS24) or two (TS27) litters, while the muscleless limb embryos came from two (TS24) or three (TS27) litters. TS24 (around e15.5 in our hands) was chosen as a stage distinct from TS23 which has already been characterised in detail (Nowlan et al., 2010; Sotiriou et al., 2019), and TS27 (around e18.5) was chosen as it is the latest reliably obtainable stage the embryos reach just before birth. Four animals per group and stage were analysed for chondrocyte morphology from the high-resolution phase-contrast synchrotron X-ray tomography data.

3D optical projection tomography and image registration

Left fore- and hind-limbs were dehydrated and stained for cartilage and mineralised tissue using alcian blue and alizarin red as previously described (Quintana and Sharpe, 2011a). Stained and fixed limbs were embedded in agarose, dehydrated and cleared in a solution of benzyl alcohol and benzyl benzoate (BABB) in preparation for 3D imaging with optical projection tomography (OPT) following prescribed protocols (Quintana and Sharpe, 2011b). Limbs were scanned under visible light to obtain
3D images of the alcian blue staining (cartilage) and under the Texas-red filter to obtain auto-fluorescent 3D images of the alizarin red stained region (the mineralised region). Scans were reconstructed using NRecon (SkyScan, Brucker microCT, 2011). Segmentation of the cartilaginous scapula, humerus, ulna, radius, pelvis, femur and tibia was performed with Mimics (v17.0, Materialise, Leuven, Belgium). The fibula was prone to deformation and twisting, making it difficult to reliably segment and was therefore not included in any analyses. As previously described (Sotiriou et al., 2019), segmentation was still possible when rudiments were fused, due to the joint line being still identifiable based on reduced intensity of staining. Segmented rudiments were prepared for image registration using TransformJ (Meijering et al., 2001) in ImageJ (Schneider et al., 2012), being first roughly aligned with other rudiments of the same type, scaled to a consistent magnification, and allocated the same canvas area. Image brightness was normalised using Matlab (version R2015a, the MathWorks, Inc., Massachusetts, USA). The Image Registration Toolkit software (Schnabel et al., 2001) (IRTK, BioMedia, Imperial College London) was used to align each rudiment to each other, with rigid registration as previously described (Sotiriou et al., 2019). A robust process of alignment of all datasets in 3D facilitated repeatable and reliable measurements of complex 3D shapes. Segmentation or image registration were not necessary for the alizarin red scans, due to the simple nature of the bone collar measurements taken.

Measurements and statistics

For joint shape characterisation, the same set of 25 forelimb measurements and 27 hindlimb measurements described in our previous study of muscleless limb joint shapes at TS23 were used, reproduced in Figures 1 and 2 (with permission). Consistent measurements were made from equivalent sections and planes of each individual rudiment by applying the same rotations to each registered rudiment dataset. Measurements were performed in Gwyddion image editing software (Gwyddion 2.44, http://gwyddion.net/, last accessed September 2021). As differences in rudiment length were previously reported for some muscleless rudiments (Nowlan et al., 2010) measurements were normalized by the length of the rudiment under investigation, in order to focus the outcomes on shape-specific (rather than overall size dependent) changes.

Rudiment lengths were measured on a lateral view. Mineralisation of the long bones was measured on a frontal cross section of the alizarin red scans. In cases where the mineralisation extent was uneven between the medial and lateral aspects, the average of the two extents was taken. Mineralisation of the pelvis and scapula was not quantified, due to difficulties in consistent measurement of the mineralisation extent in flat bones. Long bone rudiment lengths, absolute mineralisation extent, and mineralisation extent normalised to rudiment length were presented graphically.

All datasets were tested for normality with the Shapiro-Wilk test. For datasets with a normal distribution, two tailed Student’s t tests for independent samples (SPSS Statistics 24, IBM corp., Armonk, NY) were performed to determine which measurements were statistically significant between control and muscleless groups. For non-normal datasets, Mann-Whitney tests were performed. Joint shape measurements for which a significant difference ($p < 0.05$) between controls and muscleless limb mutants were found were displayed graphically, while the full table of results is available on Zenodo (https://doi.org/10.5281/zenodo.5566902). To allow for visual assessment of changes in shape, rudiment shape outlines were traced on frontal, lateral, and axial sections through the prime regions of interest for each rudiment.

Histology

Cavitation is the physical separation of the rudiments in a synovial joint which facilitates the range and extent of motion of the joint (Pacifici et al., 2005). Cavitation of the glenohumeral (shoulder),
elbow, hip and knee joints was assessed in the control and the muscleless limb embryos using standard histology. Limbs were dissected and processed for cryo-sectioning in an increased sucrose gradient (15% and 30% sucrose) as described previously (Ahmed and Nowlan, 2020). Processed limbs were embedded in OCT (optimal cutting temperature) (Agar Scientific, Stansted, UK) diluted with 50% sucrose and cut (12 µm thickness) using a cryostat (NX70, Leica Biosystems, UK). Then, frozen sections were fixed with 4% (w/v) paraformaldehyde, stained with 0.1% toluidine blue (Sigma-Aldrich) for 3 s and washed with tap water. Following air-drying, sections were imaged by transmitted illumination using a light microscope (Yenway EX30; Life Sciences Microscope, Glasgow, UK).

Chondrocyte morphological properties assessed with high resolution Synchrotron Phase contrast X-Ray Tomography

PFA-fixed embryonic limbs were imaged, submerged in ethanol, with Synchrotron Phase contrast X-Ray Tomography at the Diamond-Manchester Imaging Branchline I13–2 of the Diamond Light Source (DLS) synchrotron (Oxfordshire, United Kingdom), and the data processed and filtered as described previously (Pierantoni et al., 2021). Each image stack was imported into Materialise Mimics (Leuven, Belgium) and the distal humerus was segmented. Brightness and contrast of the image stacks of all samples were manually corrected with Fiji (Schindelin et al., 2012; Schneider et al., 2012) to the same absolute level to see the edges of cells. The 3D spot segmentation plug-in in Fiji (Ollion et al., 2013) was used to identify and label individual chondrocytes throughout the cartilaginous epiphysis.

Firstly, 3D Maxima finder was used to generate a point cloud representing the local maxima in each (putative) cell, referred to as “seeds”. The sample images and the seeds were used as inputs for the 3D spot segmentation process. A watershed was applied to define broad areas in which each cell was to be segmented. Next, the extension from local maxima to its corresponding boundary was computed using the Gaussian fit method. Concentric circles centred on the seed were calculated as a radial distribution from the local maxima, which defined the region of interest for intensity calculations around each cell. Then, a Gaussian curve was fitted to the radial distribution, and the standard deviation of the curve was used as the threshold value of a cell. Finally, voxels were clustered by means of comparing their intensity to the threshold value. A voxel was considered neighbouring to another within the same cell if its intensity was higher than the local background value, while also being lower than the intensity of the previously clustered voxel to prevent mixing voxels from cells closed to each other.

The output from 3D spot segmentation was an image stack of the same dimension as the original one, with each individual cell assigned a unique label. 3D ellipsoid fitting is another a powerful plugin in Fiji (Schindelin et al., 2012) which was used to assign an ellipsoid to each object in an image stack. The output contained measurements of each ellipsoid, including centroid position in an x-y-z space, three radii, and volume in voxels. To verify the suitability of ellipsoid fitting for quantification of the chondrocyte properties, we quantitatively compared cell volumes between the actual cell volume (as computed by the 3D spot segmentation) to the fitted ellipsoid volume for 500 ellipsoids and found no significant differences between the actual and fitted volumes.

The ellipsoid properties were imported into MATLAB (The MathWorks Inc., MA, USA) for quantification and visualisation. Overall epiphysis volume was quantified as the volume contained by the cells at the boundaries of the dataset. The volume of a cell $V_C$ was the volume of ellipsoid that was fitted, and cell number was the number of cells identified by the 3D spot segmentation process. Matrix (ECM) volume was computed by subtracting the sum of cell volumes from the epiphysis volume. Cell density was defined as the number of cells per unit volume. A cubic grid system was assigned to each sample in Matlab, with a cube dimension of 80 µm on each side. The 80 µm size was
small enough to reflect differential changes within a distal humerus and big enough to accommodate computational demands. Cell density was computed as number of cells existing in a cube divided by the epiphyseal volume within that cube. Therefore, cell density was correctly adjusted for regions at the edge of the epiphysis. One-way Analysis of Variance (ANOVA) with Levene’s test for homogeneity of variance was used to test for statistically significant differences between experimental groups (p-values of less than 0.05). The analyses were performed in SPSS. For each set of ANOVA tests, a post hoc test was carried out. If the data met the assumption of homogeneity of variance, Tukey’s HSD post hoc test was used. Otherwise, Games Howell post hoc test was used. Finally, Bonferroni methods were applied to account for multiple comparisons.

**Results**

**TS24 Forelimb**

*Glenohumeral Joint*

The muscleless limb glenoid cavity at TS24 was abnormally shaped in the lateral plane when compared to the glenoid cavity of the controls. The outlines of the glenoid cavity of the muscleless limb scapulae were more elliptical than the controls and exhibited an ectopic protrusion distally (Figure 3B, black arrow). The length-normalised height and width of the glenoid cavity in the muscleless limb embryos were significantly different to controls (Figure 4, measurements 2 and 3 respectively). Further shape abnormalities were present in the proximal humerus. The muscleless limbs had a visibly elongated humeral head in the frontal plane compared to controls (Figure 3C, purple outlines) which was reflected in a significant decrease in the major axis length of the humeral head (Figure 4, measurement 6). On the lateral plane, the shape of the muscleless limb humeri was abnormal resembling an ellipse with a tilted major axis (Figure 3D, dotted line). The glenohumeral joints of the TS24 muscleless limb embryos were fused with no clear separation between the glenoid cavity of the scapula and the humeral head (Figure 5, filled arrowhead).

*Elbow Joint*

The lateral condyle of the TS24 muscleless limb humerus had an abnormal, angular protrusion on the lateral plane (Figure 3D, asterisk) a result corroborated by the significantly greater posterior intercondylar height (Figure 4, measurement 17). The lateral condyle of muscleless limbs was also significantly wider than controls (Figure 4, measurement 12). The medial condyle of the muscleless limb humerus was irregular in the frontal plane with its shape not having the bulbous shape of the controls (Figure 3C, arrowhead). The shape of the muscleless limb distal humerus in the axial plane varied a lot from sample to sample, whereas that of the controls was more consistent between samples (Figure 3H, outlines). In the rudiments opposing the distal humerus, the muscleless limb ulnae were less intricately shaped in the lateral plane than the ulnae of the control embryos (Figure 3F, outlines) and the trochlear notch was significantly wider (Figure 4, measurement 25). The radii of the muscleless limb embryos were not different in shape to the controls and no significant differences in the shape of the proximal radii were found. Histologically, the elbow joints were fused at TS24 with no separation between the humeral condyles and the radius and ulna (Figure 5, filled arrowhead).

**TS27 Forelimb**

*Glenohumeral Joint*

At TS27, the muscleless glenohumeral joints were not as pronouncedly different in qualitative shape to the controls as at TS24. No significant differences in the TS27 glenoid cavity were detected. The glenoid cavity was elliptically shaped in both control and muscleless limb groups and only one out...
of five muscleless limb scapulae still exhibited an abnormal protrusion at the lower end of the long axis (Figure 3J, arrow). The humeral head of the muscleless limb embryos resembled that of the controls in the frontal plane (Figure 3K), but in the lateral plane, the muscleless humeral heads were visibly and quantitatively wider compared to the controls (Figure 3L; dotted line & Figure 4; measurement 8). In the axial plane (Figure 3O), the muscleless limb humeri had an indentation on the antero-lateral aspect (arrowhead) which was not present in the controls, and malformation of the humeral head was also detected though a significant difference in the humeral head axial width (Figure 4, measurement 10). Therefore, at TS27, the proximal humerus was more severely affected by the lack of muscle than the glenoid cavity. At TS27, cavitation at the glenohumeral joint had consistently occurred in the muscleless limb embryos (Figure 5B), but with a less prominent separation between the scapula and humeral head as seen in the controls (Figure 5, hollow arrowhead).

**Elbow Joint**

The shapes of the distal humerus, and proximal radius and ulna at TS27 were qualitatively similar in both control and muscleless limb embryos in the frontal and lateral planes (Figure 3K–N). However, in the axial plane, the muscleless humeral condyles appeared different to those of controls (Figure 3P), which was reflected in three significantly different measurements all made on the axial plane, namely significantly increased lateral, medial condyle and anterior intercondylar depths in the muscleless limbs compared to the controls (Figure 4, measurements 19, 20 and 23 respectively). There were no significant differences in the proximal ulna between the muscleless limb and control groups at TS27. As at TS24, no shape or size differences were observed between the radii of the two groups at TS27. The elbow joint continued to be fused at TS27 (Figure 5, filled arrowhead). Therefore, a partial recovery of elbow joint shape occurred in the muscleless limb forelimb joints by TS27, despite the lack of cavitation.

**TS24 Hindlimb**

**Hip Joint**

At TS24, the acetabulum was qualitatively and quantitatively deeper and wider in the muscleless limb group than the controls (Figure 6A, Figure 7; measurements 1 & 3). The shape of the muscleless acetabulum was also more variable in the lateral plane than in controls (Figure 6B). The muscleless limb femoral head appeared longer and more slender than in controls (Figure 6C, D) and the femoral head height was significantly greater in the muscleless limbs than in controls (Figure 7, measurement 15). These features in the muscleless proximal femur may indicate an adjustment of femoral head shape to the deeper concavity of the acetabulum in this group. At TS24, the hip joint of control limbs was clearly separated (Figure 8), while the muscleless limb hip was fused with no separation between the femoral head and the acetabulum (Figure 8, arrowhead).
Knee joint

On the frontal plane, both muscleless limb femoral condyles at TS24 had a more angular shape when compared to the condyles of the controls with the intercondylar region resembling a triangle rather than a curve (Figure 6C), reflected in a significant difference in the posterior intercondylar width (Figure 7, measurement 20). The lateral condyle of the muscleless limb tibiae protruded more prominently out from the diaphysis than controls (Figure 6E, arrowhead), reflected in a significant increase in the tibial lateral condyle height in the muscleless limb group compared to controls (Figure 7, measurement 5). Knee joints of the muscleless limb embryos was fully cavitated at TS24, as was the knee joint of the control embryos at the same stage (Figure 8).

TS27 Hindlimb

Remarkably, both the hip and the knee joints of the muscleless limb embryos at TS27 resembled in shape those of the controls (Figure 6). None of the obvious shape differences seen at TS24 remained. This was corroborated by the lack of significant differences in any of the measurements performed on the articular surfaces comprising the two joints at TS27. The hip joint of the TS27 muscleless limb embryos remained fused as no separation was seen between the femur and the acetabulum (Figure 8, arrowhead), while the muscleless limb knee joint remained fully cavitated (Figure 8). Therefore, in muscleless limb embryos at TS27 both hip and knee joint shape recovered so as to be equivalent to control shapes, despite the continued lack of cavitation in the hip joint.

Chondrocyte Morphological Properties

At least 33,000 cells per sample were morphologically quantified, as detailed in Table 1. Morphological abnormalities, and in particular the abnormal protrusion of the medial condyle, were evident in the muscleless limbs at both TS24 and TS27 (Figure 9A). Calculations of epiphyseal volume showed no significant differences in epiphyseal volume between muscleless limbs and controls at TS24, and significantly reduced volume in the muscleless limbs compared to the control limbs at TS27 (Figure 9B). While this result appears somewhat contradictory to the results for the shape feature measurements, the latter were normalised to rudiment length (which is known to be decreased when skeletal muscle is absent), while the phase contrast data was not normalised to rudiment length, as the entire rudiment was not imaged. Cell numbers were the same between TS24 and TS27, while there was a significant decrease in cell number in the TS27 muscleless limbs compared to stage matched controls (Figure 9C), correlating with trends observed for overall epiphysis volume (Figure 9B). However, matrix proportion (Figure 9D) and cell density (Figure 9E) were similar between control and muscleless limb groups at both stages, and the decrease in epiphyseal volume at TS27 is likely linked to the absolute reduction in cell number at that stage. The most pertinent temporal changes between muscleless limb and controls at the cell morphology level were in cell volume. Cell volume dramatically increased between TS24 and TS27 in both muscleless and control groups, as shown in Figure 9F. However, pertinent to the partial recovery of shape in the muscleless limbs at the later stage, cell volume in the muscleless limbs was significantly (p<0.05) smaller than in controls at TS24, but had recovered by TS27 (Figure 9F). The average (±standard deviation) cell volume at TS24 was 47.6±5.8μm$^3$ in the controls and 33.5±8.7μm$^3$ in the muscleless limbs, and at TS27, 93.1±17.9μm$^3$ in the controls and 102.1±14.0μm$^3$ in the muscleless limbs. We conclude that a recovery in cell volume in the muscleless limbs by TS27 contributes to the partial recovery in shape at the same stage.

Rudiment Length and Mineralisation

Forelimb
At TS24, the humerus, ulna and radius were all significantly shorter than controls of the same stage (Figure 10). At TS24, these three rudiments also had significantly reduced mineralisation in muscleless limbs compared to controls, for both absolute mineralisation extent, and adjusted for rudiment length (Figure 10). At TS27, the muscleless humerus and ulna were still significantly shorter than controls, while there was no significant difference between the muscleless and control radii (Figure 10). A dramatic change in mineralisation progress had occurred by TS27. Mineralisation of the three forelimb muscleless rudiments “caught up” with controls of the same age, with no significant reductions in absolute or length-proportionate mineralisation in any of the three rudiments. In fact, the proportion of mineralisation in the muscleless ulna at TS27 actually significantly exceeded that of the control ulna (Figure 10).

**Hindlimb**

At TS24, both the femur and tibia were significantly shorter in the muscleless limbs than in the controls, and the mineralisation extent of these rudiments (absolute and length-normalised) were also significantly reduced in the muscleless limbs compared to the controls (Figure 11). By TS27, there was no longer a significant difference in femoral length between the groups, while the tibia was still significantly shorter in the muscleless limbs than in the controls (Figure 11). As in the TS27 forelimb, mineralisation of the muscleless hindlimb rudiments had recovered with respect to controls of the same age, with no significant reductions in absolute or length-proportionate mineralisation relative to controls (Figure 10). As with the ulna, mineralisation of the tibia (adjusted for rudiment length) actually exceeded that of the control tibiae (Figure 11).

**Discussion**

By the latest prenatal stage (TS27/e18.5), joint shape, rudiment length and rudiment mineralisation were less severely affected by the absence of skeletal muscle than earlier in development (TS24/e15.5). Recovery of joint shape in the muscleless limbs at TS27 was more pronounced in the hindlimb than in the forelimb, with the hip and knee joint exhibiting no significant differences in measurements between the muscleless limbs and controls at TS27. Cell volume was significantly reduced in the muscleless limb distal humerus at TS24, compared to the control limbs at the same stage. However, by TS27, there was no significant differences in cell volume between muscleless limb and controls, suggesting that a recovery in cell volume is contributing, at least in part, to the recovery of muscleless limb joint morphology late in gestation. The lengths of the muscleless limb femur and radius recovered to those of the controls by TS27, while all other muscleless limb rudiments were still shorter than controls at TS27. The recovery in mineralization in the muscleless limbs over gestation was dramatic, with mineralisation of the TS27 muscleless limb long bone rudiments catching up with mineralisation in controls. Only cavitation showed limited evidence of recovery with advancing gestation in the muscleless limbs. Joints which had not cavitated at TS24 remained completely or almost completely uncavitated at TS27. Only the glenohumeral joint showed an improvement in cavitation status with advancing developmental age.

Although active muscle contractions are completely absent in the limbs of the Splotch-delayed mouse embryos, there are still two sources of mechanical stimulation acting on or in the rudiments. The first is mechanical stimulation from passive movements due to the activity of the mother and the normal littermates. We previously used computational models to predict the stresses and strains induced in the developing rudiments by a) skeletal muscle contractions and b) a very small (10µm) deflection of the limb such as would occur due to passive movements (Nowlan et al., 2012). The models predicted that the stresses induced by skeletal muscle contractions are much lower than those induced by limb movements, indicating that it is the movements of the limb that result from
skeletal muscle contraction that provide the dominant mechanical stimulation of the tissues and structures (Nowlan et al., 2012). While passive movements may have a negligible effect in normal embryos (due to the far larger active movements induced by muscle contractions), it is reasonable to believe that passive movements could play a role in the recovery of aspects of skeletal development in the muscleless limb mice. As pregnancy progresses, the space each embryo has decreases, which could increase the intensity and impact of passive stimuli in the muscleless limbs, potentially contributing to the recovery of joint shape, rudiment growth and rudiment mineralisation. The other source of mechanical stimulation acting during development, including in the muscleless limbs, is growth-generated strains and pressures. First proposed as acting like a complex field of morphogens influencing early limb morphogenesis in 2002 by Henderson and Carter, growth-generated strains and pressures arise during development when “complex configurations of connected tissues grow at different rates”, leading to “the generation of time- varying, quasi-static stresses and strains throughout the developing cells and tissues” (Henderson and Carter, 2002). During prenatal development with normal skeletal muscle and movements, growth-related strains and pressures may play a minor role, once movements have commenced. However, when skeletal muscle is absent, the biophysical stimuli arising from growth-related strains and pressures could contribute to growth, morphogenesis and differentiation in the muscleless limb skeleton.

The advance provided by the current work is it is the first demonstration of partial or full recovery of joint shape, rudiment length and rudiment mineralisation from the effects of absent skeletal muscle by the latest prenatal stage. Intriguingly, cavitation (the physical separation of the rudiments which facilitates the full range of motion of the joint) was the only aspect not to recover to some extent over development. This may indicate that cavitation is a single “rupture” type event, as previously proposed (Drachman and Sokoloff, 1966), and if movement does not occur at the critical time, the opportunity for normal cavitation is lost. It is feasible that active bending movements, localised to the joint, are needed for cavitation to occur, which would not occur even with increasing growth-generated stresses or strains due to passive movements. This concurs with our recent study, in which a cavity was artificially induced in the developing chick using an applied bending movement (Bridglal et al., 2021). What was surprising was the partial or complete recovery in joint shape despite the lack of, or incomplete, cavitation in the glenohumeral, elbow and hip joints, which contradicts our prior understanding of joint shape being heavily dependent on successful cavitation (Bridglal et al., 2021). Another interesting feature of the joint shape results was that, in the glenohumeral and elbow joints, different features were abnormal at TS27 than at TS24. This could imply that, as the joints continue to grow, the opposing surfaces are continually shaping and moulding each other, providing the opportunity for some internal correction of early abnormalities. Shape abnormalities newly arising at TS27 could be due to some form of compensation for early shape abnormalities in the opposing surface, or alternatively, particularly in the elbow joint, could be due to the continued joint fusion. However, as the hip joint did not have any quantitative shape differences at TS27, despite the ongoing lack of cavitation, this would indicate that perhaps local interaction between opposing joint surfaces is the primary driver of shape recovery, rather than the capacity for movement at the joint.

One of the most fascinating findings was the remarkable recovery of mineralisation extent in the muscleless limbs between TS24 and TS27, with two rudiments (the ulna and tibia) at TS27 actually having significantly more mineralisation (proportionate to rudiment length) compared to controls at the same age. What prompted the dramatic acceleration in mineralisation over the three day period between TS24 and TS27? Progression of the growth plates and of mineralisation has been shown to be promoted by cyclic hydrostatic pressure (Henstock et al., 2013) or applied compression loading (Khatib et al., 2021). We theorise that as the embryo grows and becomes more restricted approaching birth, the increasing levels of biophysical stimuli induced in the skeletal rudiments
could accelerate mineralisation sufficiently so that the initial delay in bone formation is recovered. Additional insights can be garnered from analyses of growth plate and mineral structure in muscleless limb and control humeri previously described by our group (Pierantoni et al., 2021). Bone volume and total volume were significantly lower in muscleless limb humeri than in controls at TS24, but there were no significant differences between the groups at TS27, corroborating the findings of the current study on humeral mineralization extent. Bone volume fraction in the humerus was similar between muscleless limb and control humeri at all stages studied, indicating that the lack of muscle may affect the rate of mineralization, rather than the actual mineral being deposited. At the level of the growth plate, there were no differences in the relative proportions of the hypertrophic and proliferating zones of the growth plate between the muscleless limb and control groups at any stage, indicating that a lack of muscle does not strongly influence the overall morphology of the growth plate. Chondrocyte volumes appeared smaller in the muscleless limb growth plates than in the controls at both TS24 and TS27, which could be a contributing factor to the sustained lower length of the humerus by TS27. Chondrocyte density appeared lower in the hypertrophic and proliferating zones of the muscleless growth plate at TS24 compared to the controls, but equivalent chondrocyte density in both regions was recovered in the muscleless limbs by TS27 (Pierantoni et al., 2021). Therefore, chondrocyte proliferation is a likely candidate for the late-gestational recovery of mineralisation in the muscleless limb mice.

The question remains as to why mineralisation in the muscleless limbs recovered to the extent of controls in all rudiments by TS27, when rudiment length only recovered in the radius and femur. The data raise the possibility that rudiment length and rudiment mineralization are mechanoregulated in different ways. The growth of the prenatal rudiment, prior to initiation of the primary ossification centre, occurs due to proliferation and expansion of the transient cartilage. After initiation of diaphyseal ossification, the transient cartilage still contributes to overall growth, evidenced by the data presented in this paper on expansion of epiphyseal chondrocyte volume between TS24 and TS27. We theorise that, in the embryonic limb, the transient chondrocytes and the growth plate chondrocytes are differentially regulated by loading from skeletal muscle. It is possible that an increase in biophysical stimuli with advancing gestational age is unable to consistently rescue rudiment length due to the rapidly decreasing quantities of transient cartilage remaining in the rudiment as development progresses. It is worth highlighting that the recovery in mineralisation extent does not necessarily mean a recovery in the ossification process, as the mineral deposited in the muscleless limbs could be abnormal or inferior in quality. Indeed, recent work from our group reported abnormal structure and localisation of key collagens in the muscleless limb cartilage and mineralised cartilage at- and prior to- TS27 (Ahmed et al., 2022), indicating indeed that the tissue does not recover by the latest prenatal stage. A useful future avenue of research would be to assess the mechanical properties of muscleless limb cartilage and mineralised cartilage, providing insight into the quality and function of the tissues of the developing limb when skeletal muscle is absent.

Surprisingly, the proportions of the muscleless ulna and tibia were actually significantly higher than in controls at TS27. The muscleless ulnae and tibiae remained significantly smaller in length than controls at TS27, and therefore a recovery in mineralisation (back to the extent of mineralisation in control ulnae and tibiae) led to the greater mineralisation proportion in the muscleless limbs. The ulna and tibia are both the dominant rudiments in the zeugopod (the middle section of the limb). They would likely be subject to lower biophysical stimuli from passive movements compared to the stylopod (most proximal region of the limb), due to greater bending moments in the stylopod. This could be a contributing factor to slower growth (and reduced length) in the muscleless limb zeugopod rudiments. Another possibility is that the accelerated mineralisation of the zeugopod is due to an increased level of growth-generated strains based on restraint between the strong stylopod
rudiment, and the complex, set of joints at the end of the stylopod at the paws, with lateral restraint due to the presence of the radius or fibula.

This study adds a novel insight into the cell-level mechanisms underlying prenatal joint growth and morphogenesis. While increase in cell volume is a well-described phenomenon underlying the expansion of the rudiment at the growth plate (when chondrocytes undergo hypertrophy) (Hunziker, 1994), we believe that our data is the first to describe a dramatic increase in cell volume, late in gestation, in the (transient) epiphyseal cartilage of the prenatal long bone rudiment. Trends in cell number were correlated with changes in overall volume across the two groups and two timepoints, but not with the recovery of shape at TS27. Average cell volumes in the muscleless limbs were approximately 30% smaller than those of the control littermates at TS24, while cell volumes were similar between the two groups by TS27. As there were no differences between muscleless limb and control groups in cell density, or in the proportion of matrix volume, at either of the stages examined, we hypothesise that cell volume is the most likely cell-level mechanism underlying the partial or full recovery of joint shape by the latest prenatal stage. Future work is planned to rigorously test this hypothesis.

There are some limitations to our research. The first is that the animal numbers are quite small, with a group size of five. However, the results were reasonably consistent, with variation within groups dwarfed by the variation between control and muscleless limb groups, as can be seen from both the raw data (dot plots) and the rudiment outlines. Our sample numbers are unfortunately insufficiently powered to test the hypothesis that the proximity or number of normally moving littermates influences skeletal development of the muscleless limb embryos. As murine embryos are sequentially arranged in the uterus, the limbs of the embryos interact with the uterine wall rather than with other embryos, and we therefore believe that the dam’s movements would have dominate the induction of passive movements. Another limitation is that our method of normalising shape measurements to rudiment length has the potential to disproportionately skew shape features which may not change in proportion to length. However, it was reassuring that significantly different measurements correlated with shape abnormalities visible in the outlines, and that shape recovery at TS27 was evident both in the qualitative and the quantitative analyses. As the Pax3 mutation is neonatal lethal, it is unfortunately not possible to quantify the effects of absent skeletal muscle on a longer timescale than in this research. Despite the dramatic improvement in the various parameters of skeletal development studied by TS27, the skeletons would not be expected to be normally functioning after birth (if, in theory, the mice would survive). The lack of cavitation in particular would inhibit movement and potentially lead to new or worsening joint shape abnormalities. Furthermore, even very slight shape abnormalities not detected by our measurements would likely impact on function and health of the joint. For example, the shape abnormalities involved in hip dysplasia are not always very dramatic, and yet with growth and over time, there are severe consequences for gait and joint health.

The relevance of the current study for human conditions is that multiple aspects of the developing murine skeleton have the capacity for recovery despite the absence of skeletal muscle and the active movements resulting from muscle contractions. Developmental dysplasia of the hip and amyoplasia result from a period of abnormal or restricted intra-uterine movement (Nowlan, 2015). Hip dysplasia is diagnosed with ultrasound after four postnatal weeks (Tan et al., 2019), and amyoplasia (despite its quite dramatic impact on skeletal morphology) is diagnosed prenatally in only 22.2% of cases (Filges and Hall, 2013). Could it be the case that the incidence of skeletal abnormalities is much greater in utero, but that majority of these have self-corrected and resolved prior to birth? Many cases of hip instability in neonates resolve without any intervention (Bialik et al., 1999), and hip dysplasia is likely more a spectrum than a dichotomous diagnosis, where potentially only the most severe
cases do not spontaneously recover either pre- or post-natally. The fact that restricted movement postnatally, for example in a cradle board (Dezateux and Rosendahl, 1997), can induce hip dysplasia in formerly healthy hips, further demonstrates the critical role of muscle, movements and mechanics in healthy skeletal development. Given that late or missed diagnoses of hip dysplasia are a significant issue, usually necessitating surgery (Broadhurst et al., 2019), it is also possible that a hip joint which satisfies the screening criteria in early postnatal life could adapt and adjust its shape negatively as the baby grows. A key question which was not answerable in this research is how the ability to self-correct and recover abnormal skeletal development declines over postnatal development. If adaptation potential of the developing skeleton declines over postnatal development, it may make sense to harness the plasticity of the developing skeleton at as young an age as possible, through physical therapy, casting or harnesses. This question will be explored in future work, with an alternative postnatal animal model.

In conclusion, skeletal development was studied at two different prenatal ages in the muscleless limb mouse. With advancing prenatal development, the effects of absent muscle on all parameters, apart from joint cavitation, become less severe. Joint shape, rudiment length, and rudiment mineralisation were significantly abnormal in multiple rudiments at TS24, but such abnormalities partially or completely recovered in all rudiments by the prenatal stage TS27. Cell-level data analyses revealed that a recovery in cell volume in the muscleless limbs over the developmental timescale examined is a likely contributor to recovery at the joint level. In contrast, joint cavitation did not recover from the lack of skeletal muscle over development. Understanding how mammalian bones and joints continue to develop in an environment without muscle contractions, but with mechanical stimulation due to the movement of the mother, provides important insights into conditions affecting human babies such as developmental dysplasia of the hip and arthrogryposis. Our animal model data would suggest that the effects of immobility in utero may reduce in severity as development progresses.

Acknowledgments

This work was funded by the European Research Council under the European Union’s Seventh Framework Programme (ERC Grant agreement number 336306) and by a a Royal Society International Exchange Grant. We thank Diamond Light Source for providing beamtime at the Diamond-Manchester Imaging Branchline I13-2 (proposal MT16557), and Andrew Bodey, Sophie Le Cann, Elin Törnquist and Aurélie Levillain for their assistance.
References


Table 1. Number of cells quantified for each sample.

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<td>69,552</td>
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<td>TS27 muscleless</td>
<td>161,111</td>
<td>163,876</td>
<td>86,338</td>
<td>75,093</td>
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</tbody>
</table>

Figure 1. Measurements made on forelimb joints. Image adapted from (Sotiriou et al., 2019) © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

Figure 2. Measurements made on hindlimb joints. Image adapted from (Sotiriou et al., 2019) © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

Figure 3. Qualitative joint shape characterisation of the forelimb joints at TS24 and at TS27. Representative 3D shapes and outlines of the scapula, humerus, radius and ulna of control (blue) and muscleless limb (purple) embryos. Arrow in B indicates abnormal protrusion of muscleless glenoid cavity, arrowhead in C represents abnormal shape of distal humerus and asterisk in D indicates abnormal shape of lateral condyle in distal humerus of muscleless limbs. Dashed line in L indicates wider appearance of humeral head in the lateral plane. Arrowhead in O indicates the abnormal shape of the muscleless limb humeral head in the axial plane.

Figure 4. Dot plots illustrating all forelimb measurements with significant differences (* p-value<0.05) between the muscleless limb (orange) and control (blue) groups at TS24 and TS27. Numbers shown before each measurement correspond to the measurements illustrated in Figure 1.

Figure 5. Cavitation in the forelimb joints at TS24 and TS27. S: scapula, H: humerus, U: ulna, R: radius. The glenohumeral joints at TS24 were not cavitated (filled arrowheads), with partial cavitation at TS27 (hollow arrowhead). The elbow joints were fused at both TS24 and TS27 (filled arrowheads). Representative images shown. The results were consistent for all three samples per stage. Scale bars 500µm.

Figure 6. Qualitative joint shape characterisation of the hindlimb joints at TS24 and at TS27. Representative 3D shapes and outlines of the pelvis, femur and tibia of control (dark blue) and muscleless limb (dark purple) embryos. Arrowhead in E indicates abnormal shape of the proximal tibia.

Figure 7. Dot plots illustrating all hindlimb measurements with significant differences (* p-value<0.05) between muscleless limb (orange) and control (blue) groups at TS24. No measurements of the hindlimb joint shapes showed any significant differences at TS27. Numbers shown before each measurement correspond to the measurements illustrated in Figure 2.

Figure 8. Cavitation in the major hindlimb joints at TS24 and TS27. P: pelvis, F: femur, T: tibia. Cavitation was absent in the hip joint at TS24 and TS27, and present in the knee joint at both stages. Representative images shown. The results were consistent for all three samples per group/stage. Scale bars 500µm.

Figure 9. Imaging using synchrotron phase contrast X-Ray tomography of muscleless limb and control distal humeri revealed cell level morphological abnormalities in the muscleless limbs. A: Outlines of shape from mid-sagittal plane of each limb imaged in 3D illustrating the shape...
abnormalities in the muscleless limb mice. Scale bar 200µm. B, C: Epiphyseal volume (B) and the number of cells (C) were similar between control and muscleless limbs at TS24, while at TS27, the muscleless limbs had a significantly reduced volume and cell numbers compared to controls. D, E: Matrix proportions (D) and cell density (E) in the distal humerus were similar between control and muscleless limb embryos at both stages examined. F: Cell volume was significantly lower in the muscleless limb mice than in controls at TS24, with no remaining significant differences in cell volume between groups by TS27. * p-value<0.05 between muscleless limb and controls within each age category.

Figure 10. Forelimb rudiment length and mineralisation. At TS24, all length and mineralisation measurements were significantly reduced in muscleless forelimbs (orange) compared to controls (blue). At TS27, humeri and ulnae were still significantly smaller in the muscleless limbs, while all mineralisation extent measures had recovered, or even exceeded, those of littermate controls. Normalised mineralisation lengths calculated by mineralised length divided by rudiment length (equivalent to mineralised proportion). *p<0.05

Figure 11. Hindlimb rudiment length and mineralisation. At TS24, femoral and tibial length and mineralisation measures were significantly reduced in muscleless limbs (orange) compared to controls (blue). At TS27, tibial lengths were still significantly lower in the muscleless limbs, while mineralisation extent measures had recovered, or even exceeded, those of littermate controls. Normalised mineralisation lengths calculated by mineralised length divided by rudiment length (equivalent to mineralised proportion). *p<0.05
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288x186mm (200 x 200 DPI)
Figure 2. Measurements made on hindlimb joints. Image adapted from (Sotiriou et al., 2019) © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.

519x494mm (79 x 79 DPI)
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492x818mm (79 x 79 DPI)
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548x699mm (79 x 79 DPI)
Figure 7. Dot plots illustrating all hindlimb measurements with significant differences (* p-value<0.05) between muscleless limb (orange) and control (blue) groups at TS24. No measurements of the hindlimb joint shapes showed any significant differences at TS27. Numbers shown before each measurement correspond to the measurements illustrated in Figure 2.

498x333mm (79 x 79 DPI)
Figure 8. Cavitation in the major hindlimb joints at TS24 and TS27. P: pelvis, F: femur, T: tibia. Cavitation was absent in the hip joint at TS24 and TS27, and present in the knee joint at both stages. Representative images shown. The results were consistent for all three samples per group/stage. Scale bars 500µm.
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1024x925mm (38 x 38 DPI)
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*p<0.05
# The ARRIVE Essential 10: author checklist

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

<table>
<thead>
<tr>
<th>Item</th>
<th>Recommendation</th>
<th>Section/line number, or reason for not reporting</th>
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<tr>
<td><strong>Study design</strong></td>
<td>For each experiment, provide brief details of study design including:</td>
<td></td>
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</table>
  a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.  
  b. The experimental unit (e.g. a single animal, litter, or cage of animals). | As described in Methods “Animal Model” |
| **Sample size** | a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.  
  b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done. | a. Methods “Animal Model”  
  b. Successfully imaged samples used. No *a priori* sample size calculations |
| **Inclusion and exclusion criteria** | a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.  
  b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.  
  c. For each analysis, report the exact value of *n* in each experimental group. | a. & c. Methods “Animal Model”. No inclusion/exclusion criteria other than genotype as stated  
  b. n/a |
| **Randomisation** | a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.  
  b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly. | a. Randomisation does not apply due to no intervention applied  
  b. confounders do not apply due to lack of intervention  
  n/a: no group allocations made |
| **Blinding** | Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis). | a. As described in Methods, “Measurements and Statistics”  
  b. n/a: outcome measure not used to determine sample size |
| **Outcome measures** | a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).  
  b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size. | a. & b. As described in Methods, “Measurements and Statistics” |
| **Statistical methods** | a. Provide details of the statistical methods used for each analysis, including software used.  
  b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met. | As described in Methods “Animal Model” |
| **Experimental animals** | a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.  
  b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures. | As described in Methods “Animal Model” (no procedures other than humane euthanasia) |
| **Experimental procedures** | For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:  
  a. What was done, how it was done and what was used.  
  b. When and how often.  
  c. Where (including detail of any acclimatisation periods).  
  d. Why (provide rationale for procedures). | Full dataset made available on Zenodo (doi provided in Methods “Measurements and Statistics”) |
| **Results** | For each experiment conducted, including independent replications, report:  
  a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).  
  b. If applicable, the effect size with a confidence interval. | |