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Biomechanical properties of bone in a mouse model of Rett syndrome

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ABSTRACT

Rett syndrome (RTT) is an X-linked genetic disorder and a major cause of intellectual disability in girls. Mutations in the methyl-CpG binding protein 2 (*MECP2*) gene are the primary cause of the disorder. Despite the dominant neurological phenotypes, *MECP2* is expressed ubiquitously throughout the body and a number of peripheral phenotypes such as scoliosis, reduced bone mineral density and skeletal fractures are also common and important clinical features of the disorder. In order to explore whether MeCP2 protein deficiency results in altered structural and functional properties of bone and to test the potential reversibility of any defects, we have conducted a series of histological, imaging and biomechanical tests of bone in a functional knockout mouse model of RTT. Both hemizygous *Mecp2*$^{stop/y}$ male mice in which *Mecp2* is silenced in all cells and female *Mecp2*$^{stop/+}$ mice in which *Mecp2* is silenced in ~50% of cells as a consequence of random X-chromosome inactivation, revealed significant reductions in cortical bone stiffness, microhardness and tensile modulus. Microstructural analysis also revealed alterations in both cortical and cancellous femoral bone between wild-type and MeCP2-deficient mice. Furthermore, unsilencing of *Mecp2* in adult mice cre-mediated stop cassette deletion resulted in a restoration of biomechanical properties (stiffness, microhardness) towards wild-type levels. These results show that MeCP2-deficiency results in overt, but potentially reversible, alterations in the biomechanical integrity of bone and highlights the importance of targeting skeletal phenotypes in considering the development of pharmacological and gene-based therapies.
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Introduction

Rett syndrome (RTT), traditionally considered a neurodevelopmental disorder, mainly affects girls and is due principally to mutations in the X-linked gene methyl-CpG-binding protein 2 (*MECP2*) [1,2]. The age of onset is typically around 6–18 months after birth with characteristic symptoms including loss of speech, reduced head growth, stereotypic hand movements, motor dysfunction and autism-like features [2]. Whilst it is well established that the majority (>95%) of classical RTT cases are due to mutations in the *MECP2* gene, the underlying function and regulation of MeCP2 protein remains unclear [3–6]. MeCP2 is a nuclear protein and is especially abundant in the brain. However, it is also expressed throughout the body [7–9] and in addition to the neurological phenotypes, a number of overt peripheral phenotypes are also common in RTT. For instance, spinal deformity (principally scoliosis and excessive kyphosis) is a very common feature, with ~50–90% of patients developing severe scoliosis [10–12], many of whom require corrective surgery. Other prominent skeletal anomalies include early osteoporosis, osteopenia, bone fractures and hip deformities [13–17]. Previous studies have found that Rett syndrome patients have reduced bone mass [18–21]. As a result, RTT patients have an increased risk of fractures and commonly sustain low-energy fractures [22]. Whilst MeCP2 is known to be expressed in bone tissues and studies have suggested a role of the protein in osteoclastogenesis [23], the role of MeCP2 in bone homeostasis is poorly defined.

The monogenic nature of RTT enables the disorder to be modelled in experimental animals. Many lines of mice have been developed in which *Mecp2* has been deleted, silenced or mutated to mimic major human mutations. These mouse lines replicate many of the features observed in RTT patients [5,24–28] and provide valuable tools for investigating MeCP2-related function/dysfunctions. An initial investigation into the skeletal system in *Mecp2*-knockout mice revealed a range of skeletal phenotypes including alterations in skeletal size, growth plate abnormalities and alternations in cortical and trabecular bone mass

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and mineralization [29]. The authors concluded that these features were consistent with an overall deficit in osteoblast function.

In the current study, we have used a range of anatomical, structural and biomechanical testing methods to investigate the biomechanical and material properties of the long bones in mice harbouring a functional knockout of *Mecp2*. Additionally, we have tested the reversibility of biomechanical phenotypes following un-silencing of the *Mecp2* gene.

Material and methods

Experimental animals

Mecp2$^{stop/+}$ mice in which the endogenous *Mecp2* allele is silenced by a targeted stop cassette (*Mecp2*^{tm2Bird}, Jackson Laboratories Stock No. 006849) were crossed with hemizygous *CreER* transgenic mice (*CAG-Cre/ESR1*, Jackson Laboratories Stock No. 004453) to create experimental cohorts [30]. A breeding strategy of crossing C57BL6/J/CBA F1 animals and using the F2 offspring was adopted as described previously [30]. The genotype of the mice was determined by polymerase chain reaction (PCR) [26]. Mice were housed in groups with littermates, maintained on a 12-h light/dark cycle and provided with food and water ad libitum. Experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and a project licence with local ethical approval under the UK Animals (Scientific Procedures) Act (1986). The unsilencing of the *Mecp2* (removal of stop cassette, henceforth known as rescue mice) was achieved by tamoxifen (100 mg/kg) administered via intraperitoneal injection following regime described previously [30]. Briefly, male mice (wild-type, *Mecp2*$^{stop/y}$ and *Mecp2*$^{stop/y}$, *CreER* (Rescue)) were given one injection of tamoxifen (100 mg/kg) per week for 3 weeks (age 6–8 weeks) followed by 4 daily injections in consecutive days in the 4th week (age 9 weeks). Mice were then culled at 14 weeks (Fig. 1). Female mice display a more delayed onset RTT-like phenotype and were given an equivalent tamoxifen treatment regimen at 18 months of age (3 weekly followed a 4 daily injections) before being culled at 20 months. Wild type control mice were treated with tamoxifen in parallel with their littermates. Samples from the same age-matched cohorts were used for imaging, biomechanical and histological tests. Mice were culled by cervical dislocation and stored frozen at −20 °C for biomechanical studies. For histological studies, mice were deeply anaesthetized with pentobarbitone (50 mg/kg, intraperitoneally) and transcardially perfused with 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). To establish MeCP2 expression in bone tissues, we used a MeCP2-GFP reporter line as described previously [31] and with sections imaged by laser scanning confocal microscopy (Bio-Rad Radiance 2100, UK).

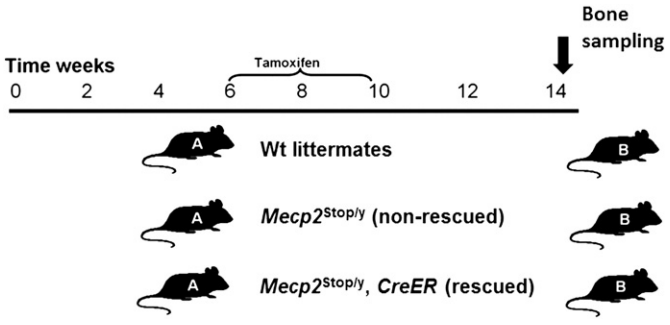

Fig. 1. Experimental design of tamoxifen regime (rescuing) of *Mecp2*$^{stop/y}$, *CreER*. Experimental design of the current study showing treatment (A) and sampling phases (B) in male mouse comparison cohorts. Wild-type (Wt), *Mecp2*$^{stop/y}$ (non rescue) and *Mecp2*-$^{stop/y}$, *CreER* (rescue) were given one injection of tamoxifen (100 mg/kg) per week for 3 weeks (age 6–8 weeks) then followed by 4 daily injections in consecutive days in the 4th week (age 9 weeks). Mice were then culled at 14 weeks and bones sampled for imaging, histology and biomechanical testing.

Specimen preparation and morphometric measurements

Both right and left femurs and tibias along with the 5th lumbar vertebrae from each mouse were carefully dissected out. Femur and tibia whole bone wet weight measurements were taken using an analytical balance (APX60, Denver Instruments, UK). The femur and tibia were imaged using a WolfVision Visualizer VZ9.4F (WolfVision Ltd., Maidenhead, UK) and gross lengths were measured using Axiovision 4.8 Software (Carl Zeiss Ltd., Cambridge, UK). Femoral length measurements were taken from the proximal aspect of the greater trochanter to the distal end of bones, along the line of the shaft. Tibial length measurement was taken from the proximal aspect of the head of the tibia to the distal most aspect of the medial malleolus. Samples were then stored at −20 °C in 0.1 M phosphate buffer prior to further testing. Right femurs were used for mechanical testing (the proximal part for the femoral neck test, the midshaft for microindentation) and left femurs were used for the bone histology (the proximal femur for sirius red and TRAP staining, the distal femur for scanning electron microscopy). Right tibias were used for μCT and three-point bending tests. The 5th lumbar vertebrae were used for bone mineral density and trabecular bone structure measures. The right humeri were used for analysis of the bone mineral structure using Small Angle X-ray Scattering (SAXS).

Micro-computed tomography (μCT)

Tibias and lumbar 5 vertebras were scanned with a SKYSCAN® 1172/A μCT Scanner (Bruker, Belgium). Images were reconstructed and analysed using the NRecon 1.6.6.0 and CT-Analyser 1.8.1.3 software (Bruker, Belgium). For the tibia, 34 μm resolution was used and the X-ray tube was operated at 54 kV and 185 μA. Bone samples were scanned in physiological 0.9% NaCl solution. For cortical bone parameter analyses, tibial 2 mm midshaft regions of interest (ROI) were selected, starting from the anatomical point of the tibiofibular junction in each specimen. A lower grey threshold value of 113 and upper grey threshold value of 255 was used as thresholding values in each cortical bone sample. Individual two dimensional object analyses were performed on six sections per specimen within each comparison genotype group to calculate the inner and outer perimeters of bone. Three dimensional analyses were further used to calculate cortical thickness, marrow area, cortical area, total area, bone volume and second moment of area.

Lumbar 5 vertebrae were scanned at a resolution of 5 μm. The X-ray tube was operated at 41 kV and 240 μA. A lower grey threshold value of 81 and upper grey threshold value of 252 was used as thresholding values in each trabecular bone sample. A cylindrical region of interest (150 slices or 0.774 mm) was selected from the centre of each vertebral body. Calibration of the standard unit of X-ray CT density from Hounsfield units to volumetric bone mineral density (vBMD) was conducted. ROIs were analysed for the following parameters: trabecular thickness, trabecular separation, trabecular bone volume, trabecular porosity, as well as degree of anisotropy (DA) and structure model index (SMI).

Mechanical tests

Right tibial and femoral shafts from each comparison group were subjected to mechanical testing (three point bending and microindentation tests respectively) after the μCT. The mechanical tests were designed to test the cortical part of bone. The tests were performed using a Zwick/Roell z2.0 testing machine (Leominster, UK) with a 100 N load cell [32].

Three-point bending test

Tibias were placed on the lower supports, at 8 mm separation, with the posterior surface of the tibia facing down. Load was applied with a loading rate of 0.1 mm s^{-1} on the shaft of the tibia using the Zwick/

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