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Thermoneutrality improves skeletal impairment in adult Prader–Willi syndrome mice

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Abstract

Human Prader–Willi syndrome (PWS) is characterised by impairments of multiple systems including the growth hormone (GH) axis and skeletal growth. To address our lack of knowledge of the influence of PWS on skeletal integrity in mice, we have characterised the endocrine and skeletal phenotype of the PWS-IC^{del} mouse model for ‘full’ PWS and determined the impact of thermoneutrality. Tibial length, epiphyseal plate width and marrow adiposity were reduced by 6, 18 and 79% in male PWS-IC^{del} mice, with osteoclast density being unaffected. Similar reductions in femoral length accompanied a 32% reduction in mid-diaphyseal cortical diameter. Distal femoral Tb.N was reduced by 62%, with individual trabeculae being less plate-like and the lattice being more fragmented (Tb.Pf increased by 63%). Cortical strength (ultimate moment) was reduced by 26% as a result of reductions in calcified tissue strength and the geometric contribution. GH and prolactin contents in PWS-IC^{del} pituitaries were reduced in proportion to their smaller pituitary size, with circulating IGF-1 concentration reduced by 37–47%. Conversely, while pituitary luteinising hormone content was halved, circulating gonadotropin concentrations were unaffected. Although longitudinal growth, marrow adiposity and femoral geometry were unaffected by thermoneutrality, strengthened calcified tissue reversed the weakened cortex of PWS-IC^{del} femora. While underactivity of the GH axis may be due to loss of *Snord116* expression and impaired limb bone geometry and strength due to loss of *Magel2* expression, comprehensive analysis of skeletal integrity in the single gene deletion models is required. Our data imply that thermoneutrality may ameliorate the elevated fracture risk associated with PWS.

Key Words

- ▶ Prader–Willi syndrome
- ▶ skeletal integrity
- ▶ pituitary insufficiency
- ▶ thermoneutrality

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Introduction

Prader–Willi syndrome (PWS) is a neurodevelopmental disorder arising from the loss of expression of one or more genes from the paternal allele of the PWS locus (Butler *et al.* 2016). The PWS phenotype is complex, characterised by neonatal hypotonia and an initial failure to thrive (Miller *et al.* 2011), the subsequent development of hyperphagia (Miller *et al.* 2011), hyperghrelinemia (Cummings *et al.* 2002), and growth hormone (GH) deficiency (Grosso *et al.* 1998), resulting in severe truncal obesity and growth retardation (Kahn *et al.* 2018).

By manipulating the murine PWS locus on chromosome 7, several mouse models for this condition have linked the contribution of individual PWS genes to specific phenotypic characteristics. For example, while loss of the *MAGE*-family gene, *Necdin* has no effect on growth or adiposity (Cattanach *et al.* 1992, Muscatelli *et al.* 2000) *Necdin*-null mice display enhanced differentiation and/or proliferation of astrocytes (Fujimoto *et al.* 2016), neocortical neural precursor cells (Minamide *et al.* 2014), haematopoietic stem cells (Asai *et al.* 2012) and pre-adipocytes (Fujiwara *et al.* 2012). Similarly, although deletion of another *MAGE*-family gene, *Magel2*, fails to induce hyperphagia with standard diets (Bischof *et al.* 2007), *Magel2*-null mice display impaired GH axis function (Tennese & Wevrick 2011) and leptin sensitivity (Pravdiviyi *et al.* 2015), accompanied by a doubling of fat mass (Bischof *et al.* 2007). In contrast, loss of the small nucleolar (sno)RNA, *Snord116*, results in mild hyperphagia and impaired meal termination, but accompanied by intra-abdominal leanness (Ding *et al.* 2008).

Such studies have revealed features of PWS not commonly reported in humans. For example, our study of metabolic homeostasis in the PWS-IC^{del} mouse, in which paternal inheritance of an imprinting centre (IC) deletion results in a complete lack of gene expression from the entire PWS interval (Chamberlain *et al.* 2004), revealed overactive brown fat and excess heat production (Golding *et al.* 2017). Unlike humans with PWS (Kahn *et al.* 2018), PWS-IC^{del} mice display profound abdominal leanness, probably resulting from a compromised capacity of PWS adipocytes to import lipid (Golding *et al.* 2017), a phenomenon reported in isolated human PWS adipocytes (Cadoudal *et al.* 2014).

Disruption of adipocyte function has extra-metabolic consequences. For example, there is a bi-directional relationship between fat and bone (Leiben *et al.* 2009), with bone marrow adipocytes and the bone-forming osteoblasts arising from the same mesenchymal stem cells (MSCs)

(Beresford *et al.* 1992, Di Iorgi *et al.* 2008) and osteogenesis being influenced by leptin (Thomas *et al.* 1999, Hamrick *et al.* 2005, Evans *et al.* 2011). Although several studies have examined the effects of the loss of specific PWS interval regions/genes on bone (Kamaludin *et al.* 2016, Khor *et al.* 2016, Baraghithy *et al.* 2019), a study of the impact of losing all of the genes in the PWS locus is lacking. We have therefore conducted a study of the growth, morphology, microarchitecture and biomechanical properties of the appendicular bones of PWS-IC^{del} mice and characterised the underlying endocrine phenotype. In addition, since we have recently shown that maintaining PWS-IC^{del} mice at thermoneutrality may reduce proportionate hyperphagia (Golding *et al.* 2017), we quantified the effect of this manipulation on bone morphology and strength.

Materials and methods

Animals

The mice used in this study were bred under the authority of the Animals (scientific procedures) Act 1986 (UK), with subsequent procedures conforming with the ARRIVE guidelines and specifically approved by the Cardiff University Animal Welfare Ethical Review Body (AWERB).

PWS-IC^{m+/p-} (referred to throughout as PWS-IC^{del}) and WT littermates were generated by crossing IC^{del}-positive males with WT females. Given that PWS-IC^{del} animals on a pure C57BL/6J background suffer severe postnatal lethality (Yang *et al.* 1998), we crossed IC^{del}-positive males with CD1 females and selectively culled WT littermates (identified on the basis of their increased size 48 h after birth) leaving only 1 or 2 WT pups per litter (Chamberlain *et al.* 2004). Animals were weaned at approximately 4 weeks of age and housed in single-sex groups with WT littermates (2–5 animals per cage).

All animals were maintained on a 12 h light/darkness cycle (lights on 07:00 h) at 20–22°C (unless otherwise stated), with *ad libitum* access to water and standard laboratory chow (Rat and Mouse No. 3 Breeding Diet, Special Diet Services Ltd., Witham, Essex, UK) containing 4.2% crude fat; 22.4% crude protein; 4.2% crude fibre; 7.6% crude ash (see Tilston *et al.* 2019 for full dietary composition).

Study 1. Tibial growth and marrow adiposity in PWS-IC^{del} mice

After an overnight fast (with water available *ad libitum*), 18-month-old male PWS-IC^{del} and WT littermates were

killed by cervical dislocation. Left tibiae were excised, the length determined with a hand-held micrometre and fixed in buffered formal saline for 48 h at 4°C before being decalcified in 0.5 M EDTA (pH 7.6). Tibiae were stored in 70% ethanol at 4°C prior to quantifying epiphyseal plate width (EPW), marrow adiposity and osteoclast number (see below).

Study 2. Femoral phenotype in PWS-IC^{del} mice

Left femora were excised from the mice in study 1; soft tissue was removed and length was measured with a hand-held micrometre. Femora were wrapped in saline-soaked gauze, snap frozen and stored at –80°C for subsequent μ -CT and biomechanical analysis (see below).

Study 3. Endocrine status in PWS-IC^{del} mice

Male and female PWS-IC^{del} and their 6–15-month-old WT littermates were anaesthetised with isoflurane and killed by decapitation. Pituitaries were dissected, weighed, snap frozen and stored at –80°C for subsequent quantification of GH, prolactin (PRL) and luteinising hormone (LH) content (see below).

Male and female PWS-IC^{del} and their 5–9-month-old WT littermates were anaesthetised with isoflurane and killed by decapitation. Pituitaries were dissected and weighed and trunk blood collected into EDTA-coated tubes, vortexed and centrifuged. Aliquots of separated plasma were snap frozen and stored at –80°C for subsequent quantification of circulating insulin-like growth factor-1 (IGF-1), LH and follicle-stimulating hormone (FSH) (see below).

Study 4. The effect of thermoneutrality on skeletal parameters in PWS-IC^{del} mice

Male and female PWS-IC^{del} and their 6–15-month-old WT littermates were group-housed in standard mouse cages (2–3 mice/cage) at 20–22°C or at thermoneutrality (30°C) (Golding *et al.* 2017). After 9 weeks, mice were anaesthetised with isoflurane and killed by decapitation. Tibiae and femora were excised and processed as above (studies 1 and 2) for subsequent quantification of growth, adiposity, geometry and strength.

Quantification of tibial epiphyseal plate width and marrow adiposity

Tibial EPWs and marrow adiposity were measured as previously described (Gevers *et al.* 2002, Thompson *et al.* 2004,

Navein *et al.* 2016, Hopkins *et al.* 2017). In brief, three 7 μ m anterior–posterior longitudinal tibial sections were stained with Masson's Trichrome and visualised under light microscopy. Total plate width was measured in triplicate on digitally captured images of each section using the interactive feature tool of Leica QWin (V3.2). Marrow adiposity was quantified on digital images of mid-diaphyseal marrow and photomicrographs analysed with National Institutes of Health (NIH) ImageJ, to quantify %adiposity, and the number and size of marrow adipocytes.

Quantification of tibial osteoclasts

To identify osteoclasts, consecutive paraffin sections were de-paraffinised, stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich), and counterstained with Mayer's haematoxylin. Histomorphometric analysis was performed on digital photomicrographs using IMAGE-PRO PLUS V.6 (Media Cybernetics, Silver Spring, MD, USA) to determine the number of TRAP⁺ osteoclasts per bone surface (N.Oc/BS).

Quantification of femoral trabecular architecture

The trabecular microarchitecture of the distal femora was assessed using a high-resolution μ -CT system (Bruker Skyscan 1272, Kontich, Belgium) as previously described in rats (Evans *et al.* 2011) and mice (Navein *et al.* 2016). Femora were thawed, mounted on the sample presentation stage and orientated by taking a series of single images. Scanning was conducted at 70 kV and 142 μ A, using a resolution of 9.04 μ m, 990 millisecond exposures, a rotation step of 0.60° and a 0.5 mm aluminium filter. Analysis was performed according to the ASBMR guidelines (Bouxsein *et al.* 2010). In brief, a 1 mm³ ROI of secondary spongiosa 0.5 mm above the centre of the distal epiphyseal plate was analysed using the CT image analysis software (CT-An; <https://www.bruker.com/products/microtomography/micro-ct-software/3dsuite.html>). Trabecular bone was separated from cortical bone within the area of interest by using the freehand drawing tool in CT-An. After scanning, femora were re-wrapped in saline-soaked gauze and re-frozen for strength testing.

Biomechanical testing

Mechanical strength of the femoral cortex was quantified by three-point bending as previously described (Stevenson *et al.* 2009, Navein *et al.* 2016), with the lower rollers set

at 6.42 and 4.04 mm apart for WT and PWS-IC^{del} femora respectively and the central roller positioned equidistant from the lower rollers over the thinnest part of the mid-diaphyseal region, to give an approximately posterior load direction. Femora were loaded at a crosshead speed of 2 mm/min until failure, with load and displacement data recorded by a Zwick Z050 tensile testing machine fitted with a 1 kN load cell (Zwick Testing Machines Ltd., Leominster, Herefordshire, UK). Ultimate tensile stress was calculated using failure load, morphometric measurements of cortical wall thicknesses and diameter (taken from cross-sectional μ -CT images corresponding to the fracture site as determined by measuring the distance from the end of the femur to the fracture point using a hand-held micrometre) and simple beam theory.

Hormone quantification

Pituitaries were homogenised in 0.5 mL lysis buffer (Tris 0.1 M pH 7.4, NaCl 0.15 M, EGTA 1 mM, EDTA 1 mM, Triton 1%, protease inhibitor cocktail (Sigma-Aldrich, P8340) and phosphatase inhibitor cocktail 3 (Sigma-Aldrich, P0044)), maintained on ice for 30 min and centrifuged for 10 min at 13,000g. Protein concentration was measured in a 1:100 dilution of 4 μ L of the supernatant with the QuantiPro BCA assay kit (Sigma-Aldrich, QPBCA-1KT) using protein standards (Sigma-Aldrich, P0914). Samples were diluted in PBS to normalise protein concentration. GH, LH and PRL levels were measured using sandwich ELISAs (Steyn *et al.* 2011, 2013, Guillou *et al.* 2015).

Plasma IGF-1 concentrations were determined in duplicate using a rat/mouse total IGF-1 immunoenzymometric assay (OCTEIA® Immunodiagnostic Systems Ltd., #AC-18F1) according to the manufacturer's instructions, with samples pre-treated to avoid binding protein interference. LH and FSH levels were measured in plasma samples using radioimmunoassay reagents provided by the National Institutes of Health (Dr A F Parlow, Torrance, CA, USA). Rat LH-I-10 and FSH-I-9 were labelled with ¹²⁵I by the chloramine-T method, and LH and FSH concentrations expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and inter-assay coefficients of variation were <8 and <10% for LH and <6 and <9% for FSH, respectively. Assay sensitivities were 5 pg/tube for LH and 20 pg/tube for FSH.

Statistical analyses

Results are expressed as mean $\pm$ S.E.M., and compared by unpaired Student's *t*-test or one-way ANOVA

and Bonferroni's selected pairs *post hoc* test (using GraphPad Prism, GraphPad Software Inc.), as indicated in the figure legends, with $P < 0.05$ considered significantly different.

Results

Study 1. Tibial growth and marrow adiposity in PWS-IC^{del} mice

Tibial length and EPW were reduced in PWS-IC^{del} males by 6% ($P < 0.001$; Fig. 1A) and 18% ($P < 0.01$; Fig. 1B) respectively. A profound (79%) reduction in tibial marrow adiposity ($P < 0.05$; Fig. 1C and inset pictures a and b) was due to a combination of a 53% reduction in marrow adipocyte number ($P < 0.05$; Fig. 1D) and a 48% reduction in mean adipocyte size ($P < 0.05$; Fig. 1E). Adipocyte size profiling (Fig. 1F) revealed a loss of larger adipocytes, especially those $> 825 \mu\text{m}^2$ ($P < 0.05$).

Analysis of TRAP⁺-stained sections revealed a 62% reduction in tibial osteoclast number ($P < 0.05$; data not shown), but when corrected for the 65% reduction in tibial trabecular surface ($P < 0.05$; data not shown), the osteoclast density was unaffected ($P = 0.403$; Fig. 1G).

Study 2. Femoral phenotype in PWS-IC^{del} mice

A 4% reduction in femoral length in PWS-IC^{del} mice ($P < 0.05$; Fig. 2A) was accompanied by a 32% reduction in cortical (anterior-posterior) diameter ($P < 0.05$; Fig. 2B) with mean cortical wall thickness in PWS-IC^{del} mice being 73% of that in WT littermates ($P = 0.055$; Fig. 2C). μ CT analysis revealed that trabecular number (Tb.N) in the distal femora of PWS-IC^{del} mice was reduced by 62% ($P < 0.01$; Fig. 2D). Although the overall trabecular thickness (Tb.Th) was unaffected ($P = 0.110$; Fig. 2E), the cross-sectional shape became more cylindrical (less plate-like) in PWS-IC^{del} mice (structural modal index (SMI) increased by 25%; $P < 0.05$; Fig. 2F). Trabecular surface was reduced in PWS-IC^{del} femora by 72% ($P = 0.0006$; data not shown), but when corrected for the 77% reduction in trabecular volume ($P = 0.0009$; data not shown), relative trabecular surface (BS/BV) was increased by 29% ($P < 0.01$; Fig. 2G). These changes were accompanied by an 18% increase in trabecular separation (Tb.Sp; $P < 0.01$; Fig. 2H) and a marked fragmentation of the trabecular lattice (63% increase in pattern factor (Tb.Pf; $P < 0.05$; Fig. 2I)). Although mean degree of anisotropy in PWS-IC^{del} mice was 125% of that in WT littermates, this index of trabecular

Figure 1

PWS-IC^{del} mice show impaired tibial growth and adiposity. (A) Quantification of tibial length in 18-month-old male WT and PWS-IC^{del} littermate mice. Tibial epiphyseal plate (EP) width (B) was quantified in Masson's Trichrome-stained sections and tibial marrow adiposity (C), marrow adipocyte number (D), size (E) and size profile (F) quantified in digital images of Toluidene blue-stained sections from WT (a) and PWS-IC^{del} (b) littermates. Osteoclast density (G) was quantified in TRAP⁺-stained sections. Data shown are mean ± S.E.M. ($n = 6$ for both genotypes), with statistical comparisons performed by Student's unpaired t -test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs WT littermates).

orientation was not significantly different ($P = 0.098$; data not shown).

Biomechanical strength of PWS-IC^{del} femoral cortex was reduced by 26% (ultimate moment; $P < 0.05$; Fig. 3A).

This was due to an 80% decrease in the geometric contribution to strength (second moment of area; $P < 0.05$; Fig. 3C), the strength of the calcified tissue (ultimate tensile stress (UTS)) being increased by 65% ($P < 0.05$; Fig. 3B).

Figure 2

PWS-IC^{del} mice show impaired femoral morphology. Measurement of femoral length (A), outer cortical (anterior-posterior) diameter (A-P Ø; B) and average cortical wall thickness (C) in 18-month-old male WT ($n = 6$ (3 for B and C)) and PWS-IC^{del} ($n = 6$) littermate mice. μ -CT was used to quantify the number (Tb.N; D), thickness (Tb.Th; E), cross-sectional shape (structural modal (SM) index; F), relative surface (BS/BV; G), separation (Tb.Sp; H) and lattice fragmentation (Pattern factor; I) of trabeculae in the distal femora. Data shown are mean ± S.E.M., with statistical comparisons performed by Student's unpaired t -test (* $P < 0.05$; ** $P < 0.01$ vs WT littermates).

Figure 3

PWS-IC^{del} mice show compromised femoral strength. Measurement of femoral strength (ultimate moment; A), tissue strength (ultimate tensile stress; B) and the geometric contribution to strength (second moment of area; C) in 18-month-old male WT ($n = 6$ for B and C) and PWS-IC^{del} ($n = 6$) littermate mice. Data shown are mean $\pm$ S.E.M., with statistical comparisons performed by Student's unpaired *t*-test (* $P < 0.05$ vs WT littermates).

Study 3. Endocrine status in PWS-IC^{del} mice

To investigate whether skeletal impairment might be due to endocrine dysfunction, we quantified pituitary and circulating hormone concentrations. Although not sexually dimorphic in either WT or PWS-IC^{del} mice, pituitary weight was reduced in both male and female PWS-IC^{del} mice by 35 and 43% respectively ($P < 0.01$ and $P < 0.001$; Fig. 4A). Similarly, pituitary GH content was reduced by 42 and 56% in male and female PWS-IC^{del} mice ($P < 0.05$; Fig. 4B) in proportion to protein content (data not shown). While average pituitary PRL content in male PWS-IC^{del} mice was only 45% of that in WT males, this was not significantly different ($P > 0.05$). In contrast, female PWS-IC^{del} mice showed a 41% reduction in PRL content ($P < 0.05$; Fig. 4C); the marked sexual dimorphism seen in WT mice ($P < 0.0001$) being retained in PWS-IC^{del} littermates ($P < 0.01$; Fig. 4C). This sexual dimorphism ($P < 0.0001$), but not PRL deficiency, was retained when PRL contents were corrected for protein content (data not shown). Male PWS-IC^{del} mice showed a marked (58%) reduction in pituitary LH content ($P < 0.0001$; Fig. 4D), but while mean LH content in female PWS-IC^{del} mice was only 54% of that in WT females, this was not significantly different ($P = 0.535$; Fig. 4D). In addition, the marked sexual dimorphism in LH content seen in WT mice ($P < 0.0001$) was not replicated in PWS-IC^{del} littermates ($P = 0.412$). These differences in LH content remained after correction for protein content ($P < 0.05$; data not shown).

Circulating IGF-1 was reduced in male and female PWS-IC^{del} mice by 47 and 37% respectively ($P < 0.0001$ and $P < 0.001$; Fig. 5B). Although mean plasma LH and FSH concentration in PWS-IC^{del} males were 163 and 123% of that in male WT littermates, these were not significantly different ($P > 0.900$; Fig. 5C and D). Plasma LH and FSH concentrations were comparable in WT and PWS-IC^{del} females, and there was no sexual dimorphism in circulating gonadotrophin levels in either genotype (Fig. 5C and D).

Study 4. The effect of thermoneutrality on skeletal parameters in PWS-IC^{del} mice

As in study 1, tibial length in male PWS-IC^{del} mice at standard ambient temperature was reduced by 11% ($P < 0.0001$; Fig. 6A), with a similar (10%) reduction in females ($P < 0.0001$; data not shown). This difference was maintained at thermoneutrality in males (9% reduction; $P < 0.001$; Fig. 6A) and females (8% reduction; $P < 0.0001$), thermoneutrality having no effect on either tibial length or EPW in either genotype (Fig. 6A and B).

Mean tibial marrow adiposity and adipocyte number in PWS-IC^{del} mice at standard ambient temperature were only 22 and 29% of that in WT males, but given the variation in the WT data, these were not statistically different ($P = 0.5668$ (adiposity); Fig. 6C; $P = 0.3388$ (adipocyte number); Fig. 6D). Thermoneutrality had no statistically

Figure 4

PWS-IC^{del} mice show multiple pituitary hormone deficiencies. Quantification of pituitary weight (A) and growth hormone (GH; B), prolactin (PRL; C) and luteinising hormone (LH; D) contents in 6–15-month-old male and female WT ($n = 6$) and PWS-IC^{del} ($n = 6$ (male) and 5 (female)) littermate mice. Data shown are mean $\pm$ S.E.M., with statistical comparisons performed by 1-way ANOVA and Bonferroni *post hoc* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ vs WT littermates (same sex); †† $P < 0.01$; †††† $P < 0.0001$ vs male littermates (same genotype)).

Figure 5

PWS-IC^{del} mice show reduced GH-IGF-1 axis activity. Quantification of pituitary weight (A) and plasma insulin-like growth factor-1 (IGF-1; B), luteinising hormone (LH; C) and follicle-stimulating hormone (FSH; D) in 5–9-month-old male and female WT and PWS-IC^{del} ($n = 6$ per group) littermate mice. Data shown are mean $\pm$ S.E.M., with statistical comparisons performed by 1-way ANOVA and Bonferroni *post hoc* test (A and B) or Kruskal–Wallis test (C and D) (**** $P < 0.0001$; *** $P < 0.001$ vs WT littermates (same sex); **** $P < 0.0001$ vs male littermates (same genotype)).

significant effect on tibial marrow adiposity (Fig. 6C) or adipocyte size in either WT or PWS-IC^{del} males (Fig. 6E). Parallel results were also obtained in females (data not shown). Analysis of the adipocyte size profile revealed that

while differences were seen between PWS-IC^{del} males and their WT littermates at room temperature (e.g. there were less adipocytes in the size range 525–572 μm^2 in PWS-IC^{del} mice (Fig. 6F; $P = 0.038$)), these differences were abolished in mice maintained at thermoneutrality (Fig. 6G).

As above, femoral length and cortical diameter were reduced by 8 and 25% in male PWS-IC^{del} mice at 20–22°C ($P < 0.0001$; Fig. 7A and B), with average cortical wall thickness not being significantly different (Fig. 7C). None of these geometric variables were altered by increasing the ambient temperature to thermoneutrality (Fig. 7A, B and C). However, the 48% reduction in the biomechanical strength of the femoral cortex in PWS-IC^{del} mice at room temperature ($P < 0.0001$; Fig. 7D), was abolished when PWS-IC^{del} mice were maintained at thermoneutrality (Fig. 7D). This improvement in biomechanical performance was entirely due to the significant increase in the strength of the calcified tissue, UTS in PWS-IC^{del} mice at 30°C being 91% higher than that in WT littermates at thermoneutrality ($P < 0.01$; Fig. 7E). In the absence of any significant effect of thermoneutrality on femoral geometry, there was no change in the geometric contribution to strength, which remained at 32% of that in WT mice (Fig. 7F). Similar results were obtained in females, the impaired biomechanical strength in PWS-IC^{del} mice at 20–22°C ($P = 0.007$), being ameliorated at thermoneutrality ($P = 0.215$), as a consequence of the contribution of tissue

Figure 6

Thermoneutrality has little effect on growth and marrow adiposity in PWS-IC^{del} mice. Tibial length (A), epiphyseal plate (EP) width (B), marrow adiposity (C), marrow adipocyte number (D) and mean adipocyte size (E) were quantified in 6–15-month-old male WT and PWS-IC^{del} littermate mice after being maintained at either standard ambient temperature (20–22°C) or thermoneutrality (30°C) for 9 weeks. Adipocyte size profiles are presented for standard ambient temperature (F) and thermoneutrality (G). Data shown are mean $\pm$ S.E.M. ($n = 6$ (room temperature) and 5 (thermoneutrality)), with statistical comparisons performed by 1-way ANOVA and Bonferroni *post hoc* test (A–E; **** $P < 0.0001$ vs room temperature (same genotype)) or unpaired Student's *t*-test (F and G; * $P < 0.05$ vs WT littermates (same temperature)).

Figure 7

Thermoneutrality alleviates impaired femoral strength in PWS-IC^{del} mice. Femoral length (A), cortical anterior-posterior diameter (A-P Ø; B), average cortical wall thickness (C), cortical strength (ultimate moment; D), tissue strength (ultimate tensile stress; E) and the geometric contribution to strength (second moment of area; F) were quantified in 6–15-month-old male WT and PWS-IC^{del} after being maintained at either standard ambient temperature (20–22°C) or thermoneutrality (30°C) for 9 weeks ($n = 6$ (room temperature) and 5 (thermoneutrality)). Data shown are mean $\pm$ S.E.M., with statistical comparisons performed by 1-way ANOVA and Bonferroni *post hoc* test (** $P < 0.01$; **** $P < 0.0001$ vs WT littermates (same ambient temperature)).

strength, the impaired geometric contribution being exacerbated ($P = 0.006$) (data not shown).

Discussion

Loss of gene expression from the paternal allele of chromosome 15q11-q13 results in the marked disturbances in neural development, hormone secretion and metabolic homeostasis that characterise PWS. However, despite impaired GH secretion and GH replacement long being considered a key feature of this condition and an important element in therapeutic strategy (Lee *et al.* 1987, Deal *et al.* 2013, Carias & Wevrick 2019), our understanding of the significance of GH deficiency for skeletal growth and integrity in the preclinical animal models of PWS is surprisingly superficial. To address this gap in our knowledge, we have analysed the phenotype of the weight-bearing long bones of the PWS-IC^{del} mouse model for 'full' PWS, shedding new light on the mechanisms of fracture risk in this complex condition.

Three prominent features of the observed skeletal phenotype deserve comment: impaired morphometric growth, impaired marrow adiposity and impaired biomechanical strength.

Preliminary evidence of growth retardation has been reported in most of the murine models for PWS, including mice with uniparental disomy (Cattanach *et al.* 1992) and deletions of *Snrpn-Ube3a* (Tsai *et al.* 1999a), *Snurf/Snrpn* exon 2 (Tsai *et al.* 1999b), *Snord116* (Ding *et al.* 2008) and *Magel2* (Bischof *et al.* 2007, Baraghithy *et al.* 2019), with *Necdin*^{del} mice showing normal growth (Tsai *et al.* 1999a). However, initial attempts to quantify skeletal growth

following IC deletion, in which expression of all the genes in the PWS locus is lost, have been hampered by high neonatal mortality (Yang *et al.* 1998). Having developed a breeding strategy to partially overcome this problem, we now report that PWS-IC^{del} mice display consistent shortening of appendicular bones.

This growth impairment is most likely to result from the marked deficiency in the GH-IGF-1 axis (40–50% reductions in both pituitary GH content and circulating IGF-1). Although we cannot exclude a potential reduction in GH sensitivity, it is evident from comparison with other murine models for isolated GH deficiency (GH-D) or reduced GH signalling that the degree of growth retardation in mice appears to reflect the severity of axis inactivation, with complete loss of GH secretion/signalling producing the most severe phenotype (20–25% reduction in body length; Zhou *et al.* 1997, Alba & Salvatori 2004, Stevenson *et al.* 2009).

It is important to note, however, that femoral diameter (reduced by 32% in PWS-IC^{del} mice) was more profoundly affected than longitudinal growth. This occurred without affecting cortical wall thickness. Although broadly similar findings in mice with reduced GH signalling (Stevenson *et al.* 2009) suggest that loss of GH activity may be an important determinant, the fact that cortical diameter is only reduced by 17% in the complete absence of GH receptors implies that other factors in the PWS endotype may contribute to this diminution of diameter.

While GH deficiency may be the most likely cause, we cannot exclude the potentially negative influence of gonadotropin deficiency on bone formation (Yarram *et al.* 2003). In contrast, the observed PRL deficiency is unlikely to represent a significant factor in this context as PRL has been shown to inhibit osteoblast function

(Cross *et al.* 2000). However, given the growing evidence for impaired oxytocin signalling in mouse models for PWS (Schaller *et al.* 2010), further analysis should investigate the potentially negative impact of oxytocin loss on the skeletal phenotype (Elabd *et al.* 2008).

A potential physical mechanism relates to the marked reduction in body weight (reduced by 40%) and adiposity (individual fat pad weights reduced by 67–84%) seen in PWS-IC^{del} mice (Golding *et al.* 2017). This leanness has a number of consequences. Firstly, the loading forces being applied to these weight-bearing bones are significantly reduced. These forces promote the remodelling of the bone to enhance diameter and weight-bearing capacity (David *et al.* 2007, Luu *et al.* 2009). Although muscle mass was not quantified in the current study, muscle hypoplasia in the *Magel2*^{del} mouse model for PWS/Schaaf–Yang syndrome (SYS) (Kamaludin *et al.* 2016), indicates that this could represent a possible transduction mechanism. Secondly, such profound reductions in abdominal fat mass are likely to cause a dramatic reduction in circulating leptin. Any effect of hypoleptinaemia is likely to be enhanced by changes in the marrow milieu resulting from the equally dramatic reduction in marrow adiposity in this model.

This marked decline in tibial marrow adiposity is due to reductions in both marrow adipocyte number and size. While the latter parallels the changes we previously reported in intra-abdominal white adipose tissue (Golding *et al.* 2017), our current data indicate that in the bone marrow at least, impaired adipogenesis is also a significant factor. In the context of the barrage of endocrine signals promoting marrow adiposity, this is quite remarkable. For example, *dw/dw* rats, which show a similar degree of GH-D accompanied by intra-abdominal leanness, show elevated marrow adiposity (mainly increased adipogenesis) (Gevers *et al.* 2002), with GH treatment inhibiting adipogenesis and triglyceride storage (Gevers *et al.* 2002). In addition, since ghrelin is powerfully adipogenic in bone marrow (Thompson *et al.* 2004, Davies *et al.* 2009, Hopkins *et al.* 2017), the marked hyperghrelinaemia in PWS-IC^{del} mice (Golding *et al.* 2017) should elevate marrow adiposity. Clearly, the anti-adipogenic signals in PWS-IC^{del} mice are more than sufficient to reverse these influences. The absence of the larger adipocytes in bone marrow corresponds with the reported impairment of lipid storage capacity in intra-abdominal WAT in these mice (Golding *et al.* 2017) and the impairment of lipid storage in cultured adipocytes from humans with PWS (Cadoudal *et al.* 2014). Whether the obesity that usually accompanies PWS in humans leads to parallel changes in marrow adiposity remains to be established.

With this degree of leanness in the marrow, it is highly likely that the production of leptin from marrow adipocytes (Laharrague *et al.* 1998) is reduced in parallel. Interestingly, intra-bone marrow infusion of leptin in GH-D rats not only halves marrow adiposity by suppressing adipogenesis, but increases osteoblast surface (Evans *et al.* 2011). Given this role of leptin in maintaining the bone microenvironment, one would expect bones from PWS-IC^{del} mice to show evidence of elevated osteoblast activity. However, while the function of PWS-IC^{del} osteoblasts should be examined *in vitro*, our data indicate that osteoblast activity does not appear to be enhanced *in vivo*. Indeed, the combination of unaltered relative trabecular surface, a more fragmented trabecular lattice and an unchanged osteoclast density, imply that PWS-IC^{del} osteoblast number and/or activity is reduced. The combined reduction in adipocytes and osteoblasts is unusual and suggests a failure in the proliferation of MSCs or their subsequent differentiation.

In the context of this endocrine and cellular milieu, the biomechanical integrity of the femoral cortex is clearly compromised. Surprisingly, UTS, a measure of the strength of the calcified tissue, *per se*, is significantly increased. Such increases in tissue strength usually result from a greater density of either matrix proteins or hydroxyapatite. This is likely to be due to the reduction in GH axis activity, producing slower growing and less remodelled bone (Locatelli & Bianchi 2014). Nevertheless, despite this increased tissue strength, the geometric component of strength (second moment of area) is profoundly reduced, which corresponds directly with the smaller cortical diameter discussed earlier. Indeed, the impairment of this geometric component is more than sufficient to translate an elevated UTS into a compromised overall organ strength.

While the analysis of single gene deletion models in this context is far from complete, the information available suggests some potential genetic mechanisms underlying the complex skeletal phenotype observed. The impairment of the GH axis may be due in part to the loss of expression of *Snord116*, because although *Snord116*^{del} mice show normal pituitary volume, somatotroph number (Ding *et al.* 2008) and GH content (Burnett *et al.* 2017), circulating IGF-1 is reduced by 60–70% (Ding *et al.* 2008, Qi *et al.* 2016). This lack of GH action, possibly as the result of impaired activity of the hormone pro-convertase enzyme PC1 (Burnett *et al.* 2017) increases GH-releasing hormone mRNA expression in the arcuate nucleus (Qi *et al.* 2016) reflecting impaired GH feedback. In contrast, male *Magel2*^{del} mice show normal IGF-1 levels, with IGF-1 secretion and ghrelin-induced

(but not GHRH-induced) GH responses impaired in female mice (Tennese & Wevrick 2011). However, given the episodic nature of GH secretion in rodents, establishing the relationship between these specific genes and the parameters of spontaneous GH secretion would be more readily achieved in a larger species.

In the context of skeletal growth, body length is only modestly reduced in *Snord116*^{del} mice, with a 10% reduction in bone mineral density (Ding *et al.* 2008, Qi *et al.* 2016). Although overall body length is normal in the absence of *Magel2* (Bischof *et al.* 2007), femoral length, cortical diameter and cortical wall thickness are reduced in female *Magel2*^{del} mice by 9–13% (Baraghithy *et al.* 2019). Indeed, this is the only model in which a comprehensive analysis has been made of the skeletal phenotype. Interestingly, although these mice also show comparable reductions in trabecular number, trabecular fragmentation, femoral strength and UTS to that reported here in the PWS-IC^{del} mice, marrow adiposity is more than doubled (Baraghithy *et al.* 2019) compared to the profound reduction reported here. This implies that loss of one of the other genes in the PWS locus either disrupts the relationship between adipocyte and osteoblast differentiation, or the proliferation of MSCs. Since *Necdin* has already been identified as a regulator of astrocyte (Fujimoto *et al.* 2016), neocortical neural precursor cell (Minamide *et al.* 2014), haematopoietic stem cell (Asai *et al.* 2012) and pre-adipocyte (Fujiwara *et al.* 2012) differentiation, this seems like a potential candidate.

Given that the normal relationship between fat mass and bone remodelling is disrupted in PWS-IC^{del} mice, and our previous evidence that raising ambient temperature suppresses brown adipose tissue function (Golding *et al.* 2017), we investigated the effects of maintaining PWS-IC^{del} mice at thermoneutrality on this altered skeletal phenotype. While this manipulation had no effect on marrow adiposity, there was a significant improvement in biomechanical strength as a result of an increased strength of the calcified tissue. This is remarkable since we have previously shown that this manipulation halved food intake in PWS-IC^{del} mice (Golding *et al.* 2017). When coupled with evidence that thermoneutrality normalises skeletal length and bone mineral density in *Snord116*^{del} mice (Qi *et al.* 2017), this implies that bone turnover is dramatically reduced at thermoneutrality. This interpretation is supported by evidence that thermoneutrality increases bone formation and reduces bone resorption in growing female C57BL/6J mice, while dramatically reducing food intake and doubling marrow adiposity (Iwaniec *et al.* 2016). The latter observation

serves to re-emphasise the likely impairment of adipocyte function in the PWS-IC^{del} model (Golding *et al.* 2017).

In summary, our data show that the longitudinal growth and biomechanical integrity of long bones are markedly impaired in the PWS-IC^{del} mouse model for 'full' PWS. Whether this impairment is matched by deficits in the biomechanical properties of other types of bone, for example calvarial or vertebral bone, is yet to be established, but our data not only provide a biomechanical basis for the increased fracture risk in PWS (Butler *et al.* 2002, Longhi *et al.* 2015), but indicate that thermoneutrality may be beneficial in this context. The final phenotype observed in the PWS-IC^{del} mice appears to result from the combined loss of several genes from within the PWS locus, but a more precise genetic cause for the individual aspects remains to be fully elucidated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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