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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-ICdel 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-ICdel 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-ICdel 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-ICdel 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), hyperghrelinæmia (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 (Pravdivyi 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 (Cadould 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, Baragithy 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 ms X 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
Skeletal phenotype in PWS-IC del mice. In brief, 1 mm del Gevers (2004) and mice (Navein et al., 2016). After 9 weeks, mice were 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 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 handheld 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 7µm 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 70kV and 142 µA, using a resolution of 9.04 µm, 990 millisecond exposures, a rotation step of 0.60° and a 0.5mm 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<sup>del</sup> males 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 13,000 g. Protein concentration was measured in a 1:100 dilution of 4 µL 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 (OCYTEIA® Immunodiagnostics 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 ± 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 < 0.05 considered significantly different.

**Results**

**Study 1. Tibial growth and marrow adiposity in PWS-IC<sup>del</sup> mice**

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

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

**Study 2. Femoral phenotype in PWS-IC<sup>del</sup> mice**

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

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Biomechanical strength of PWS-IC<sup>del</sup> 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).
Study 3. Endocrine status in PWS-IC<sup>del</sup> 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<sup>del</sup> mice, pituitary weight was reduced in both male and female PWS-IC<sup>del</sup> 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<sup>del</sup> mice (P<0.05; Fig. 4B) in proportion to protein content (data not shown). While average pituitary PRL content in male PWS-IC<sup>del</sup> mice was only 45% of that in WT males, this was not significantly different (P>0.05). In contrast, female PWS-IC<sup>del</sup> 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<sup>del</sup> litters (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<sup>del</sup> mice showed a marked (58%) reduction in pituitary LH content (P<0.0001; Fig. 4D), but while mean LH content in female PWS-IC<sup>del</sup> 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<sup>del</sup> litters (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<sup>del</sup> 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<sup>del</sup> males were 163 and 123% of that in WT males, these were not significantly different (P>0.900; Fig. 5C and D). Plasma LH and FSH concentrations were comparable in WT and PWS-IC<sup>del</sup> 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<sup>del</sup> mice

As in study 1, tibial length in male PWS-IC<sup>del</sup> 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<sup>del</sup> 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 significant effect on these parameters (P>0.05; data not shown). In addition, mean tibia cross-sectional area was reduced by 11% in male PWS-IC<sup>del</sup> mice at standard ambient temperature, but thermoneutrality maintained this parameter (P<0.0001).}

Figure 3

PWS-IC<sup>del</sup> 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) and PWS-IC<sup>del</sup> (n = 6) litters. Data shown are mean ± s.e.m., with statistical comparisons performed by Student’s unpaired t-test (*P < 0.05 vs WT littermates).

Figure 4

PWS-IC<sup>del</sup> 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<sup>del</sup> (n = 6 (male) and 5 (female)) litters. Data shown are mean ± 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 litters (same sex); †††P < 0.001; ††††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<sup>Del</sup> males (Fig. 6E).

Analysis of the adipocyte size profile revealed that while differences were seen between PWS-IC<sup>Del</sup> males and their WT littermates at room temperature (e.g. there were less adipocytes in the size range 525–572 µm<sup>2</sup> in PWS-IC<sup>Del</sup> 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<sup>Del</sup> 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<sup>Del</sup> mice at room temperature (P < 0.0001; Fig. 7D), was abolished when PWS-IC<sup>Del</sup> 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<sup>Del</sup> 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<sup>Del</sup> mice at 20–22°C (P = 0.007), being ameliorated at thermoneutrality (P = 0.215), as a consequence of the contribution of tissue...
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 Snurp-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 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-ICdel 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 Magel2del 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 hyperghrelinæmia in PWS-ICdel mice (Golding et al. 2017) should elevate marrow adiposity. Clearly, the anti-adipogenic signals in PWS-ICdel 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-ICdel mice to show evidence of elevated osteoblast activity. However, while the function of PWS-ICdel 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-ICdel 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 Snord116del 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 Magel2del mice show normal IGF-1 levels, with IGF-1 secretion and ghrelin-induced
(but not GHRH-induced) GH responses impaired in female mice (Tennessee & Wervick 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 Snord116del 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 Magel2del 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-ICdel 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-ICdel 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-ICdel 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-ICdel mice (Golding et al. 2017). When coupled with evidence that thermoneutrality normalises skeletal length and bone mineral density in Snord116del 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-ICdel 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-ICdel 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-ICdel 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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