Thermoneutrality improves skeletal impairment in adult Prader-Willi syndrome mice

Thomas M Braxton1, Dionne EA Sarpong1, Janine L Dovey1, Anne Guillou2, Bronwen AJ Evans3, Juan M Castellano4, Bethany E Keenan5, Saja Baraghithy6, Sam L Evans5, Manuel Tena-Sempere4,7, Patrice Mollard2, Joseph Tam6 and Timothy Wells1*

1School of Biosciences, Cardiff University, Museum Avenue, Cardiff, CF10 3AX, UK
2IGF, CNRS, INSERM, University of Montpellier, Montpellier, France
3School of Medicine, Cardiff University, Cardiff CF14 4XN, UK
4Physiology Section, Faculty of Medicine, University of Cordoba, and Instituto Maimonides de Investigacion Biomedica de Cordoba (IMBIC), 14004 Cordoba, Spain
5School of Engineering, Cardiff University, The Parade, Cardiff, CF24 3AA, UK
6Obesity and Metabolism Laboratory, Institute for Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem 9112001, Israel
7CIBER Fisiopatologia de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, 14004 Cordoba, Spain

Running head: Skeletal Phenotype in PWS-ICdel Mice

Word Count: 4986 (including the Abstract)

*Author for correspondence:

Dr Timothy Wells
School of Biosciences,
Cardiff University,
Cardiff. CF10 3US, UK
Tel: (+44) 2920 874977
Fax: (+44) 2920 876328
E-mail: wellst@cardiff.ac.uk
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<sup>del</sup> 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<sup>del</sup> 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<sup>del</sup> pituitaries were reduced in proportion to their smaller pituitary size, with circulating IGF-1 concentration reduced by 37-47%. Conversely, while pituitary LH content was halved, circulating gonadotropin concentrations were unaffected. Although longitudinal growth, marrow adiposity and femoral geometry were unaffected by thermoneutrality, strengthened calcified tissue reversed weakened cortex of PWS-IC<sup>del</sup> 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.
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), hyperghrelinaemia (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), hematopoietic 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-ICdel 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<sup>del</sup> 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 (Khor et al., 2016, Kamaludin 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<sup>del</sup>
mice and characterised the underlying endocrine phenotype. In addition, since we have
recently shown that maintaining PWS-IC<sup>del</sup> 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<sup>m+p-</sup> (referred to throughout as PWS-IC<sup>del</sup>) and wild-type (WT) littermates were generated by crossing IC<sup>del</sup>-positive males with WT females. Given that PWS-IC<sup>del</sup> animals on a pure C57BL/6J background suffer severe postnatal lethality (Yang et al, 1998), we crossed IC<sup>del</sup> positive males with CD1 females and selectively culled WT littermates (identified on the basis of their increased size 48 hours 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 12hr light/dark cycle (lights on 07:00h) 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<sup>del</sup> mice

After an overnight fast (with water available ad libitum), 18-month old male PWS-IC<sup>del</sup> and WT littermates were killed by cervical dislocation. Left tibiae were excised, the
length determined with a hand-held micrometer and fixed in buffered formal saline for
48hrs at 4°C before being decalcified in 0.5M 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<sup>del</sup> mice
Left femora were excised from the mice in study 1, soft tissue removed and length
measured with a hand-held micrometer. 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<sup>del</sup> mice
Male and female PWS-IC<sup>del</sup> 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 growth hormone (GH),
prolactin (PRL) and luteinising hormone (LH) content (see below).

Male and female PWS-IC<sup>del</sup> 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<sup>del</sup> mice
Male and female PWS-IC<sup>del</sup> 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) (<span>Golding et al, 2017</span>). After 9 weeks, mice were anaesthetised with isoflurane and killed by decapitation. Tibiae and femora were excised and processed as above (studies 1 & 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 (<span>Gevers et al, 2002; Thompson et al, 2004, Navein et al, 2016, Hopkins et al, 2017</span>). 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) Image J, 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) to determine the number of TRAP<sup>+</sup> 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 142µA, using a resolution of 9.04µm, 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 1mm³ ROI of secondary spongiosa 0.5mm above the centre of the distal epiphyseal plate was analyzed 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 and 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> 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 2mm/min until failure, with load and displacement data recorded by a Zwick Z050 tensile testing machine fitted with a 1kN load cell (Zwick Testing Machines Ltd., Leominster, United Kingdom). 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 micrometer) and simple beam theory.
**Hormone Quantification**

Pituitaries were homogenized in 0.5ml lysis buffer (TRIS 0.1M pH 7.4, NaCl 0.15M, EGTA 1mM, EDTA 1mM, Triton 1%, Protease inhibitor cocktail (Sigma-Aldrich, P8340) and Phosphatase inhibitor cocktail 3 (Sigma- Aldrich, P0044)), maintained on ice for 30 mins and centrifuged for 10 mins at 13000g. Protein concentration was measured in a 1:100 dilution of 4µl 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 normalize protein concentration. GH, LH and PRL levels were measured using sandwich ELISAs ([Steyn et al., 2011; Steyn et al., 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 labeled with $^{125}$I 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 ± SEM, and compared by unpaired Student’s t-test or 1-way ANOVA and Bonferroni’s selected pairs *post hoc* test (using GraphPad Prism, GraphPad Software Inc., San Diego. CA., USA), as indicated in the figure legends, with $P < 0.05$ considered significantly different.
Results

Study 1. Tibial growth and marrow adiposity in PWS-ICdel mice

Tibial length and EPW were reduced in PWS-ICdel 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 & 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 >825µm² (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-ICdel mice

A 4% reduction in femoral length in PWS-ICdel 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-ICdel mice being 73% of that in WT littermates (P=0.055;
Fig 2C). µCT analysis revealed that trabecular number (Tb.N) in the distal femora of
PWS-ICdel 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-ICdel mice (structural modal index (SMI)
increased by 25%; P<0.05; Fig 2F). Trabecular surface was reduced in PWS-ICdel
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<sup>del</sup> mice was 125% of that in WT littermates, this
index of trabecular orientation was not significantly different (P=0.098; data not shown).

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> 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<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> 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<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 male WT littermates, these were not significantly different (P>0.900; Fig 5C & 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 & 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 were 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 nor EPW in either genotype (Fig 6A & 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 tibial marrow adiposity (Fig 6C) or adipocyte size in either WT or PWS-IC<sup>del</sup> 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<sup>del</sup> males and their WT littermates at room temperature (e.g. there were less adipocytes in the size range 525-572µm<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 & 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-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 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\textsuperscript{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 \textit{Snrpn-Ube3a} (Tsai et al, 1999a), \textit{Snurf/Snrpn exon 2} (Tsai et al, 1999b), \textit{Snord116} (Ding et al, 2008) and \textit{Magel2} (Bischof et al, 2007; Baraghithy, 2019), with \textit{Necdin}\textsuperscript{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\textsuperscript{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; Alba and Salvatori, 2004; Zhou et al, 1997; Stevenson et al, 2009).

It is important to note, however, that femoral diameter (reduced by 32% in PWS-ICdel 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\textsuperscript{del} mice (Golding \textit{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 \textit{et al}, 2007; Luu \textit{et al}, 2009). Although muscle mass was not quantified in the current study, muscle hypoplasia in the \textit{Magel2}\textsuperscript{del} mouse model for PWS/Schaaf-Yang syndrome (SYS) (Kamaludin \textit{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 \textit{et al}, 2017), our current data indicates 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, \textit{dw/dw} rats, which show a similar degree of GH-D accompanied by intra-abdominal leanness, show elevated marrow adiposity (mainly increased adipogenesis) (Gevers \textit{et al}, 2002), with GH treatment inhibiting adipogenesis and triglyceride storage (Gevers \textit{et al}, 2002). In addition, since ghrelin is powerfully adipogenic in bone marrow (Thompson \textit{et al}, 2004; Davies \textit{et al}, 2009; Hopkins \textit{et al}, 2017), the marked hyperghrelinaemia in PWS-IC\textsuperscript{del} mice (Golding \textit{et al}, 2017) should elevate marrow adiposity. Clearly, the anti-adipogenic signals in PWS-IC\textsuperscript{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<sup>del</sup> mice to show evidence of elevated osteoblast activity. However, while the function of PWS-IC<sup>del</sup> osteoblasts should be examined <i>in vitro</i>, our data indicate that osteoblast activity does not appear to be enhanced <i>in vivo</i>. Indeed, the combination of unaltered relative trabecular surface, a more fragmented trabecular lattice and an unchanged osteoclast density, imply that PWS-IC<sup>del</sup> 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, <i>per se</i>, 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 above. 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\textsuperscript{del} mice show normal pituitary volume, somatotroph number (Ding \textit{et al}, 2008) and GH content (Burnett \textit{et al}, 2017), circulating IGF-1 is reduced by 60-70% (Ding \textit{et al}, 2008; Qi \textit{et al}, 2016). This lack of GH action, possibly as the result of impaired activity of the hormone pro-convertase enzyme PC1 (Burnett \textit{et al}, 2017) increases GH-releasing hormone mRNA expression in the arcuate nucleus (Qi \textit{et al}, 2016) reflecting impaired GH feedback. In contrast, male Magel2\textsuperscript{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\textsuperscript{del} mice, with a 10% reduction in bone mineral density (Ding \textit{et al}, 2008; Qi \textit{et al}, 2016). Although overall body length is normal in the absence of Magel2 (Bischof \textit{et al}, 2007), femoral length, cortical diameter and cortical wall thickness are reduced in female Magel2\textsuperscript{del} mice by 9-13% (Baraghithy \textit{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\textsuperscript{del} mice, marrow adiposity is more than doubled (Baraghithy \textit{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 \textit{Necdin} has already been identified as a regulator of astrocyte (Fujimoto \textit{et al}, 2016), neocortical neural precursor cell (Minamide \textit{et al}, 2014), hematopoietic stem cell (Asai \textit{et al}, 2012) and pre-adipocyte (Fujiwara \textit{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\textsuperscript{del} mice, and our previous evidence that raising ambient temperature suppresses brown adipose tissue function (Golding \textit{et al}, 2017), we investigated the effects of maintaining PWS-IC\textsuperscript{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\textsuperscript{del} mice (Golding \textit{et al}, 2017). When coupled with evidence that thermoneutrality normalises skeletal length and bone mineral density in Snord116\textsuperscript{del} mice (Qi \textit{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 C17BL/6J mice, while dramatically reducing food intake and doubling marrow adiposity (Iwaniec \textit{et al}, 2016). The latter observation serves to re-emphasize the likely impairment of adipocyte function in the PWS-IC\textsuperscript{del} model (Golding \textit{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, e.g. calvarial or vertebral bone, has 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.
References


Copyright © 2019 Society for Endocrinology


Evans BAJ, Bull MJ, Kench RC, Fox RE, Morgan LD, Stevenson AE, Gevers EF, Perry MJ & Wells T 2011 The influence of leptin on trabecular architecture and marrow...


Thomas T, Gori F, Khosla S, Jensen MD, Burguera B & Riggs BL 1999 Leptin acts on human marrow stromal cells to enhance differentiation to osteoblasts and to inhibit differentiation to adipocytes. *Endocrinology* 140:1630-1638. (doi: [https://doi.org/10.1210/endo.140.4.6637](https://doi.org/10.1210/endo.140.4.6637))


Tsai TF, Armstrong D & Beaudet AL 1999a Necdin-deficient mice do not show lethality or the obesity and infertility of Prader-Willi syndrome. *Nature Genetics* 22 15-16. (doi: [10.1038/8722](https://doi.org/10.1038/8722))


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.

Acknowledgments

The authors thank JBIOS staff and Derek Scarborough (Cardiff University) for excellent technical support.

Funding

This work was supported by a summer studentship awarded by the Foundation for Prader-Willi Research (to TMB, BEK and TW). AG and PM were supported by grants from the Agence Nationale de la Recherche (ANR-15-CE14-0012-01, ANR-18-C14-0017-01), Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, and Université de Montpellier.
Figure Legends

Figure 1: PWS-IC<sub>del</sub> mice show impaired tibial growth and adiposity. Quantification of tibial length (A), in 18-month old male WT (n=6) and PWS-IC<sub>del</sub> (n=6) littermate mice. Tibial epiphysial 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<sub>del</sub> (b) littermates. Osteoclast density (G) was quantified in TRAP<sup>+</sup>-stained sections. Data shown are mean ± SEM (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).

Figure 2: PWS-IC<sub>del</sub> 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 & C)) and PWS-IC<sub>del</sub> (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 ± SEM, with statistical comparisons performed by Student’s unpaired T-test (*P<0.05; **P<0.01 vs WT littermates).

Figure 3: PWS-IC<sub>del</sub> 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 (3 for B & C)) and PWS-IC<sub>del</sub> (n=6) littermate mice. Data shown are mean ±
SEM, 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 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)) littermate mice. Data shown are mean ± SEM, 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<sup>del</sup> 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<sup>del</sup> (n=6 per group) littermate mice. Data shown are mean ± SEM, with statistical comparisons performed by 1-way ANOVA and Bonferroni post hoc test (A & B) or Kruskal-Wallis test (C & D) (**P<0.01; ****P<0.0001 vs WT littermates (same sex); †††† P<0.0001 vs male littermates (same genotype)).

**Figure 6:** Thermoneutrality has little effect on growth and marrow adiposity in PWS-IC<sup>del</sup> 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<sup>del</sup> 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 ± SEM (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 & G; * P<0.05 vs WT littermates (same temperature)).

Figure 7: Thermoneutrality has little effect on growth and marrow adiposity in PWS-IC\textsuperscript{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\textsuperscript{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 ± SEM, 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)).
Figure 1: PWS-IC\textsuperscript{del} mice show impaired tibial growth and adiposity. Quantification of tibial length (A), in 18-month old male WT (n=6) and PWS-IC\textsuperscript{del} (n=6) littermate mice. Tibial epiphysial plate (EP) width (B) was quantified in Masson’s Trichrome-stained sections and tibial marrow adiposity (C), adipocyte number (D), size (E) and Size profile (F) quantified in digital images of Toluidene Blue-stained sections from WT (a) and PWS-IC\textsuperscript{del} (b) littermates. Osteoclast density (G) was quantified in TRAP\textsuperscript{+} stained sections. Data shown are mean ± SEM (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).
Figure 2: PWS-IC<sup>del</sup> 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 & C)) and PWS-IC<sup>del</sup> (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 ± SEM, with statistical comparisons performed by Student’s unpaired T-test (*P<0.05; **P<0.01 vs WT littermates).
Figure 3: PWS-ICdel 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 (3 for B & C)) and PWS-ICdel (n=6) littermate mice. Data shown are mean ± SEM, 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 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)) littermate mice. Data shown are mean ± SEM, 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<sup>del</sup> 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<sup>del</sup> (n=6 per group) littermate mice. Data shown are mean ± SEM, with statistical comparisons performed by 1-way ANOVA and Bonferroni post hoc test (A & B) or Kruskal-Wallis test (C & D) (**P<0.001; ****P<0.0001 vs WT littermates (same sex); †††† P<0.0001 vs male littermates (same genotype)).
Figure 6: Thermoneutrality has little effect on growth and marrow adiposity in PWS-IC\textsuperscript{del} mice. Tibial length (A), epiphyseal plate (EP) width (B), marrow adiposity (C), adipocyte number (D) and mean adipocyte size (E) were quantified in 6-15-month old male WT and PWS-IC\textsuperscript{del} 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 ± SEM (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 & G; * P<0.05 vs WT littermates (same temperature)).
Figure 7: Thermoneutrality has little effect on growth and marrow adiposity in PWS-IC<sup>del</sup> mice. Tibial length (A), epiphyseal plate (EP) width (B), marrow adiposity (C), adipocyte number (D) and mean adipocyte size (E) were quantified in 6-15-month old male WT and PWS-IC<sup>del</sup> 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 ± SEM, 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)).