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# Endogenous glucocorticoid signalling in osteoblasts is necessary to maintain normal bone structure in mice ☆

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ABSTRACT

The role of endogenous glucocorticosteroids (GC) in bone development is ill-defined. Using the Col2.3-11βHSD2 transgenic (tg) mouse model, we examined the effect of osteoblast-targeted disruption of intracellular GC signalling on bone growth and strength, and its modulation by factors such as age, gender and skeletal site.

Tibiae and L3 vertebrae of 3 and 7-week-old, male and female wild type (WT) mice and their tg, age and sex matched littermates were analysed by micro-CT and mechanical testing. Data were analysed separately for 3 and 7-week-old mice by 2-way ANOVA using genotype (WT, tg), gender and their interactions as factors.

Transgenic mice were characterised by lower bone volume, lower trabecular number and higher trabecular separation in tibial trabecular bone, as well as lower tibial cortical bone area and periosteal and endosteal perimeters. These changes resulted in a marked decrease in mechanical bone strength and stiffness in sexually mature, 7-week-old mice. In the tibia, the observed transgene effect was present in 3 and 7-week-old animals, indicating that the biological effect of disrupted GC signalling was independent of sexual maturity. This was not the case for the vertebral bones, where significant differences between tg and WT mice were seen in 7 but not in 3-week-old animals, suggesting that the effects of the transgene at this site may be modulated by age and/or changes in circulating sex hormone levels.

Taken together, our results demonstrate that endogenous glucocorticoids may be required for normal bone growth but that their effect on bone structure and strength varies according to the skeletal site and sexual maturity of the animals.



## Introduction

Glucocorticoid-induced osteoporosis is a serious adverse effect of long-term exogenous or endogenous hypercortisolism [1–3]. About 50% of all patients treated with glucocorticoids (GC) for ≥6 months will suffer significant bone loss. In most patients treated with high-dose GC, markers of bone formation are suppressed, reflecting the profound effects of GC on osteoblasts (OB), while markers of bone resorption are often increased [4–6]. Histologically, mean wall thickness is usually decreased, reflecting the reduced amount of bone replaced in each remodeling unit [7]. Thus, while GC appear to exert their effects on the skeleton through a number of pathways (e.g. suppression of sex steroids, muscle wasting, changes in renal/intestinal calcium handling), it appears that the most important catabolic skeletal actions of GC directly target the OB.

These *catabolic* effects of GC on bone are in direct contrast to their known *anabolic* actions. Osteoblasts and their precursors are highly responsive to GC, which promote osteoprogenitor proliferation, lineage commitment, osteoblast differentiation and bone development [8–13]. These apparent disparities in GC action on bone are not fully explained by dosing or experimental design, signifying that the *mechanisms* by which GC exert their effects on cells and tissues are poorly understood. In fact, neither the primary cellular target for GC, nor the function of GC in normal bone modeling and remodeling are clearly defined.

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Kream and co-workers generated a transgenic mouse in which the rat gene for a glucocorticoid inactivating enzyme, 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) was linked to the 2.3 kb of the rat collagen type I (Col2.3) promoter (Col2.3-11βHSD2) [10,14]. This results in the targeted overexpression of 11βHSD2, and the inactivation of endogenous GC exclusively in mature osteoblasts and osteocytes. Of note, 11βHSD2 is absent from skeletal tissues under physiological conditions and mostly expressed in the kidney. Accordingly, 11βHSD2 is not found to be expressed in the calvaria, femur or vertebra of non-transgenic (WT) littermates [10,12,13]. This model provides a unique opportunity to study the role of endogenous GC signalling in bone development and maintenance. Thus, sexually mature transgenic female (but not male) Col2.3-11βHSD2 tg mice have been shown to exhibit vertebral (but not femoral) trabecular osteopenia [10], while femoral cortical bone parameters were decreased in both female and male mice [11]. While these data suggest that GC signalling in osteoblasts may be required to maintain normal bone mass in sexually mature mice, it remains unclear whether age, gender, sexual maturity and skeletal site determine the skeletal response to osteoblastic GC signalling in mice. The purpose of the present study was therefore to determine the role of endogenous GC in bone growth and maintenance, and its dependence on age, sexual maturity, gender and skeletal site. To this aim, we compared bone structure and strength of both the tibiae and L3 vertebrae of sexually immature, 3-week-old and sexually mature, 7-week-old male and female WT mice and their Col2.3-11βHSD2 tg age and sex matched littermates.

## Materials and methods

### *Experimental design, tissue collection and specimen preparation*

Col2.3-11βHSD2 transgenic (tg) mice were generated as described previously [10,14] and were provided as a gift by Dr Barbara Kream (Dept. of Medicine, University of Connecticut Health Center, Farmington, CT, USA). Mice were maintained at the animal facilities of the ANZAC Research Institute (Sydney, Australia) in accordance with Institutional Animal Welfare Guidelines and according to an approved protocol.

Tibiae and L3 vertebrae of 3 and 7-week-old, male and female wild type (WT) mice and their tg, age and sex matched littermates ($n = 10$ per group) were harvested for microcomputed tomography (micro-CT). A separate group of animals was used for mechanical testing of tibia bone strength ($n = 4$–10 per group).

The tibiae and lumbar vertebrae of each mouse were dissected and fixed in 4% paraformaldehyde, buffered with 0.1 mol/L phosphate buffer (pH 7.4), for 48 h at 4 °C and then kept in phosphate buffered saline until micro-CT.

### *Microcomputed tomography (Micro-CT)*

Micro-CT of the tibiae and lumbar vertebrae (L3) was performed using a Skyscan 1172 scanner (SkyScan, Kontich, Belgium). Scanning was done at 100 kV, 100 μA using a 1 mm aluminium filter and exposure set to 590 ms. In total, 1800 projections were collected at a resolution of 6.93 μm/pixel. Reconstruction of sections was achieved using a modified Feldkamp cone-beam algorithm with beam hardening correction set to 50%. 'CTAnalyser' software (ver. 1.02, SkyScan) was employed for morphometric quantification of trabecular and cortical bone indices.

Transverse sections of tibial cortical bone were analysed at a level 20% by length below the proximal end of the tibia, level, corresponding to ~2.4 mm in 3-week-old mice, and to ~3 mm in 7-week-old mice from the distal surface of the proximal growth plate. Parameters of tibial trabecular bone were measured in a defined inter-endosteal area located 0.5 to 1.5 mm below the growth plate. The entire volume of trabecular bone within the vertebral body was selected for analysis of vertebral trabecular bone indices. Trabecular morphology was described by measuring bone volume fraction [BV/tissue volume (TV)], trabecular number (Tb.N.), trabecular separation (Tb.Sp.),

**Table 1**
Structural and mechanical bone parameters in 7-week-old mice.

| | | 7-week-old mice | | | | | | | | 2-way ANOVA | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean values (SD) | | | | | | | | *p*-values | | | | |
| | | Female | | | | Male | | | | % change tg vs. WT | | Main effects | | Interaction |
| | | WT | | tg | | WT | | tg | | F | M | Genotype | Gender | Gender × genotype |
| | Body weight (g) | 27.0 | (1.4) | 25.0 | (2.9) | 34.6 | (1.8) | 30.7 | (2.1) | −7 | −12 | <0.001 | <0.001 | ns |
| Tibia | BV/TV (%) | 13.1 | (1.9) | 10.3 | (2.6) | 16.2 | (2.6) | 11.8 | (2.9) | −22 | −27 | 0.001 | 0.007 | ns |
| | Tb.N. (1/mm) | 2.44 | (0.35) | 2.03 | (0.48) | 3.18 | (0.49) | 2.45 | (0.61) | −17 | −23 | 0.001 | 0.001 | ns |
| | Tb.Sp. (μm) | 228 | (26) | 271 | (32) | 181 | (23) | 231 | (50) | 19 | 27 | 0.001 | 0.001 | ns |
| | Tb.Th. (μm) | 53.5 | (0.8) | 50.9 | (1.9) | 50.9 | (1.6) | 48.5 | (2.1) | −5 | −5 | <0.001 | <0.001 | ns |
| | BS/BV | 69 | (2) | 75 | (4) | 71 | (3) | 78 | (4) | 10 | 9 | <0.001 | 0.028 | ns |
| | Tb.Pf (1/mm) | 23.0 | (2.4) | 26.7 | (3.8) | 22.3 | (2.1) | 26.9 | (3.1) | 16 | 21 | <0.001 | ns | ns |
| | Cortical bone area (mm2) | 1.04 | (0.06) | 0.92 | (0.07) | 1.08 | (0.06) | 0.90 | (0.08) | −11 | −16 | <0.001 | ns | ns |
| | Periosteal perimeter (mm) | 6.80 | (0.30) | 6.35 | (0.20) | 7.36 | (0.28) | 6.73 | (0.30) | −7 | −9 | <0.001 | <0.001 | ns |
| | Endosteal perimeter (mm) | 4.45 | (0.32) | 4.09 | (0.26) | 5.17 | (0.41) | 4.41 | (0.39) | −8 | −15 | <0.001 | <0.001 | ns |
| | Cortical thickness (μm) | 166 | (14) | 156 | (10) | 152 | (19) | 145 | (10) | −6 | −4 | ns | 0.008 | ns |
| | Tibia length (mm) | 17.1 | (0.2) | 16.8 | (0.4) | 17.5 | (0.5) | 17.0 | (0.6) | −2 | −3 | 0.002 | ns | ns |
| | Maximum load (N) | 13.3 | (1.64) | 10.2 | (1.08) | 14.4 | (1.75) | 10.0 | (1.57) | −23 | −31 | <0.001 | ns | ns |
| | Bending modulus (Stiffness) | 613 | (87) | 455 | (88) | 674 | (142) | 417 | (106) | −26 | −38 | <0.001 | ns | ns |
| Vertebra | BV/TV (%) | 21.6 | (2.9) | 18.2 | (3.6) | 19.9 | (2.6) | 15.6 | (2.4) | −16 | −22 | 0.001 | 0.023 | ns |
| | Tb.N. (1/mm) | 3.99 | (0.41) | 3.42 | (0.46) | 3.89 | (0.4) | 3.18 | (0.45) | −14 | −18 | <0.001 | ns | ns |
| | Tb.Sp. (μm) | 195 | (8) | 215 | (11) | 174 | (11) | 200 | (19) | 10 | 15 | <0.001 | <0.001 | ns |
| | Tb.Th. (μm) | 53.9 | (2.5) | 53.0 | (3.6) | 51.2 | (2.7) | 48.9 | (2.0) | −2 | −4 | ns | 0.001 | ns |
| | BS/BV | 66 | (5) | 70 | (7) | 73 | (5) | 77 | (4) | 5 | 6 | 0.033 | 0.001 | ns |
| | Tb.Pf (1/mm) | 10.1 | (3.6) | 14.1 | (3.7) | 13.2 | (2.8) | 18.3 | (2.6) | 39 | 38 | <0.001 | 0.001 | ns |

Abbreviations: WT = wild type, tg = transgenic, F = female, M = male, BV/TV = bone volume fraction (bone volume/tissue volume), Tb.N. = trabecular number, Tb.Sp. = trabecular separation, Tb.Th. = trabecular thickness, BS/BV = bone surface to bone volume ratio, Tb.Pf = trabecular pattern factor (index of connectivity − the higher value of Tb.Pf the lower connectivity).
Data are shown as mean (±SD) and percent change in tg vs. WT mice. Results of a full-factorial 2-way ANOVA as main effects of genotype and gender are presented. No influence of gender on genotype effect (gender × genotype interaction) was observed in ANOVA.

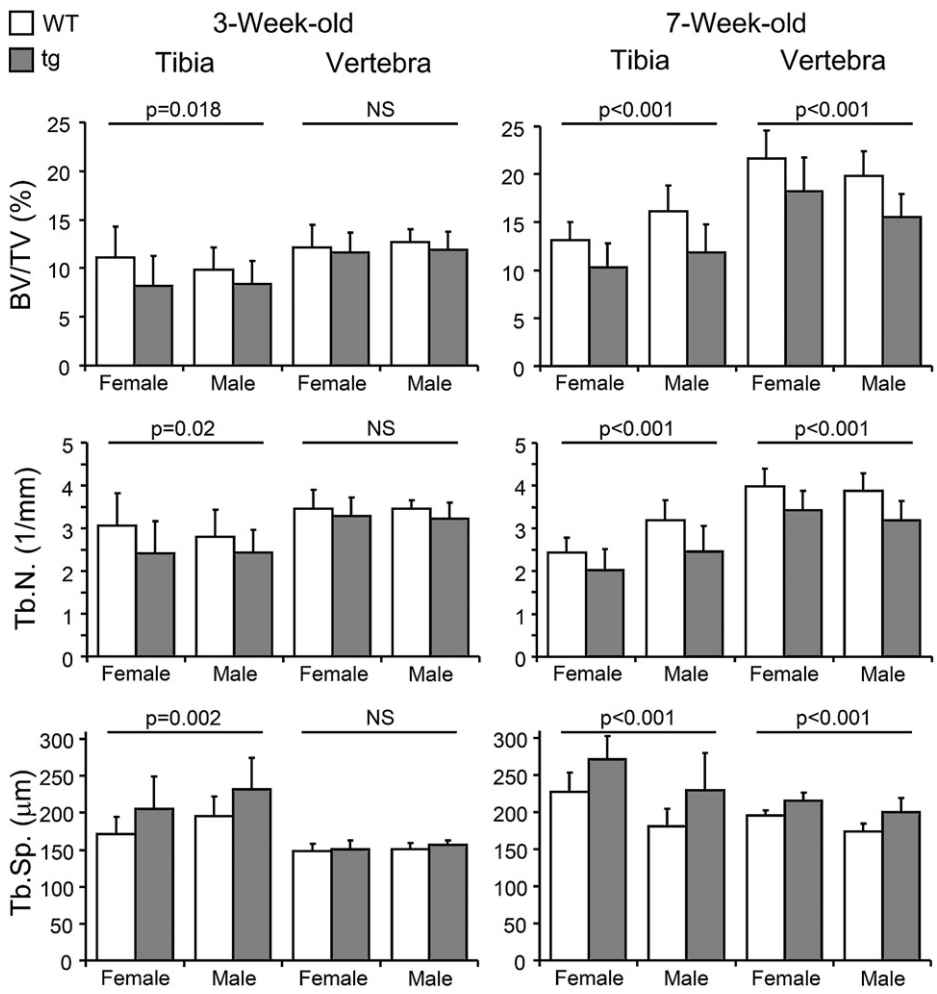


**Fig. 1.** Bone volume fraction, trabecular number and trabecular separation of tibial and vertebral trabecular bone. Data are shown as means ± SD. *p*-values are for genotype effects by 2-way ANOVA with gender and genotype as factors. WT — wild type, tg — transgenic, BV/TV — bone volume fraction (bone volume/tissue volume), Tb.N. — trabecular number, Tb.Sp. — trabecular separation.

trabecular thickness (Tb.Th.), bone surface to bone volume ratio (BS/BV) and trabecular pattern factor (Tb.Pf.) which is an index of trabecular connectivity (the higher value of Tb.Pf. the lower connectivity) [15].

*Faxitron analysis of tibia length*

Tibia length was measured from digital X-ray images (MX-50 X-ray cabinet, Faxitron) of bones aligned next to each other in same orientation using interactive image analysis software (ImageJ ver. 1.37, National Institutes of Health, USA).

*Mechanical testing*

Bone strength was measured in three-point bending, using an Instron 5566 testing machine (Instron Pty Ltd, Bayswater Victoria, Australia) with a 100N load cell and a custom designed bending rig with a 10 mm span. The crosshead speed was 5 mm/min for all samples. All of the bones were tested with the central loading pin placed on the anterior midshaft of the tibia. All testing and data acquisition was controlled by Instron Bluehill software, version 2.5. Tests were carried out until failure, defined as an 80% drop in measured load over 100 ms. Peak load and extension at failure were recorded, and bending modulus was calculated as a least squares fit to the linear portion of the load-extension curve.

*Statistical analysis*

The main aim of statistical analysis was to determine the effects of the Col2.3-11βHSD2 transgene (i.e. the disruption of endogenous GC signalling in osteoblasts and osteocytes) on bone in 3 and 7-week-old mice at two distinct skeletal sites: tibia and vertebra, and to assess possible interactions of the transgene effects with gender. To this aim, full-factorial 2-way ANOVA was conducted with transgene (WT, tg) and gender (male, female) as factors separately for 3 and 7-week-old

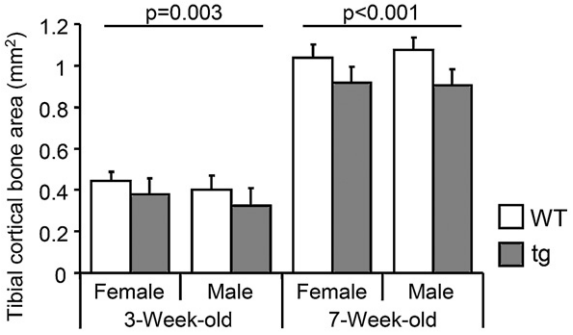


**Fig. 2.** Tibial cortical bone area. Data are shown as means ± SD. *p*-values are for genotype effects by 2-way ANOVA with gender and genotype as factors. WT — wild type, tg — transgenic.

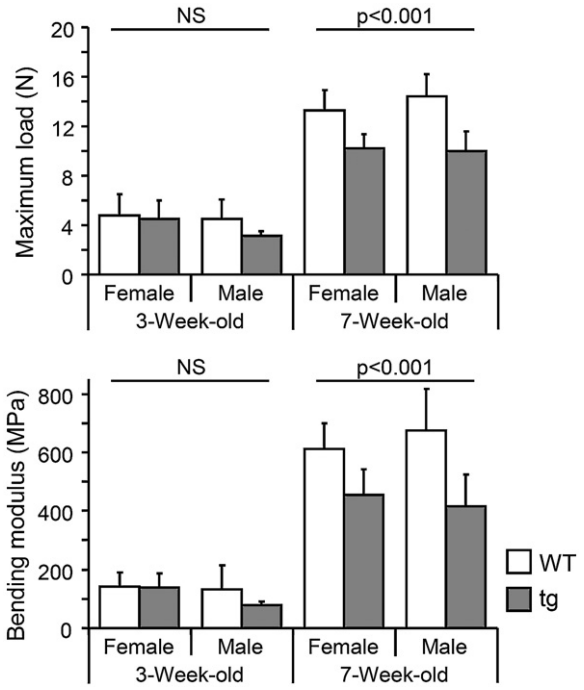


**Fig. 3.** Tibia mechanical bone strength. Data are shown as means ± SD. *p*-values are for genotype effects by 2-way ANOVA with gender and genotype as factors. Group sizes were as follows: 3-week-old mice: $n = 4$–5; 7-week-old mice: $n = 10$. WT — wild type, tg — transgenic.

mice. Contrast analysis was used for comparisons between individual groups [16].

Due to the observed difference in body weight between WT and tg animals, all structural parameters were analysed by ANCOVA in the same way as ANOVA, with body weight as a covariate. All statistical analyses were performed using an SPSS statistics program (SPSS ver. 15, SPSS Inc. Chicago, USA). A level of $p<0.05$ was considered significant.

## Results

### *Bone structure and mechanical strength in sexually mature mice*

We first assessed bone morphology in sexually mature, 7-week-old mice. Compared to WT mice, tibial trabecular bone volume fraction (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th.) were all significantly reduced in Col2.3-11βHSD2 tg mice ($p<0.001$ each), while trabecular separation (Tb.Sp.) and pattern factor (Tb.Pf) were significantly increased ($p<0.001$ each) (Table 1, Fig. 1). Analogous changes of similar magnitude were observed when analysing the vertebra of Col2.3-11βHSD2 tg mice, although trabecular thickness was not changed significantly when compared to WT mice (Table 1). When comparing Col2.3-11βHSD2 tg with WT mice, cortical bone area, and periosteal and endosteal perimeters were significantly decreased ($p<0.001$ each), while cortical thickness was similar in Col2.3-11βHSD2 tg and WT animals (Table 1, Fig. 2). Tibial length was slightly decreased in tg mice ($p = 0.002$ vs. WT).

Both maximum load and bending modulus were significantly decreased in tg animals when compared to WT animals ($p<0.001$ each), indicating lower bone strength and stiffness in tg mice (Table 1, Fig. 3).

Gender-specific differences were apparent for most of the trabecular bone parameters in sexually mature animals (Table 1). In cortical bone, differences in bone geometry were apparent in that males exhibited both larger periosteal and endosteal perimeters as well as lower cortical thickness than female mice. This resulted in similar cortical bone areas and mechanical properties in male and female mice. However, despite such gender-specific differences in bone structure and geometry, no interaction between gender and genotype was found for any of the analysed parameters, indicating that the effect of GC signalling on bone structure in sexually mature mice is similar in male and female animals.

**Table 2**
Structural and mechanical bone parameters in 3-week-old mice.

| | | 3-week-old mice | | | | | | | | 2-way ANOVA | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean values (SD) | | | | | | | | *p*-values | | | | |
| | | Female | | | | Male | | | | % change tg vs. WT | | Main effects | | Interaction |
| | | WT | | tg | | WT | | tg | | F | M | Genotype | Gender | Gender × genotype |
| | Body weight (g) | 12.7 | (1.1) | 10.5 | (1.8) | 12.2 | (1.5) | 9.7 | (2.0) | −18 | −21 | <0.001 | ns | ns |
| Tibia | BV/TV (%) | 11.1 | (3.2) | 8.2 | (3.1) | 9.8 | (2.3) | 8.4 | (2.3) | −26 | −15 | 0.018 | ns | ns |
| | Tb.N. (1/mm) | 3.07 | (0.75) | 2.41 | (0.75) | 2.80 | (0.64) | 2.42 | (0.54) | −21 | −14 | 0.02 | ns | ns |
| | Tb.Sp. (μm) | 171 | (23) | 206 | (43) | 196 | (26) | 233 | (42) | 20 | 19 | 0.002 | 0.025 | ns |
| | Tb.Th. (μm) | 35.5 | (2.2) | 33.4 | (2.8) | 35.3 | (1.9) | 34.5 | (2.6) | −6 | −2 | ns | ns | ns |
| | BS/BV | 117 | (14) | 134 | (22) | 120 | (11) | 125 | (15) | 14 | 4 | 0.046 | ns | ns |
| | Tb.Pf (1/mm) | 31.2 | (7.8) | 39.6 | (11.7) | 31.4 | (7.1) | 33.2 | (7.5) | 27 | 6 | ns | ns | ns |
| | Cortical bone area (mm2) | 0.45 | (0.04) | 0.38 | (0.08) | 0.40 | (0.07) | 0.32 | (0.09) | −14 | −19 | 0.003 | 0.028 | ns |
| | Periosteal perimeter (mm) | 4.97 | (0.20) | 4.60 | (0.61) | 4.72 | (0.27) | 4.28 | (0.41) | −8 | −9 | 0.003 | 0.032 | ns |
| | Endosteal perimeter (mm) | 4.10 | (0.15) | 3.68 | (0.57) | 3.91 | (0.19) | 3.45 | (0.37) | −10 | −12 | 0.001 | ns | ns |
| | Cortical thickness (um) | 112 | (14) | 109 | (17) | 101 | (15) | 95 | (19) | −3 | −6 | ns | 0.016 | ns |
| | Tibia length (mm) | 12.7 | (0.3) | 12.2 | (0.7) | 12.3 | (0.5) | 11.8 | (0.7) | −4 | −5 | 0.007 | ns | ns |
| | Maximum load (N) | 4.8 | (1.73) | 4.5 | (1.5) | 4.5 | (1.54) | 3.1 | (0.36) | −6 | −30 | ns | ns | ns |
| | Bending modulus (Stiffness) (MPa) | 143 | (46) | 138 | (49) | 133 | (80) | 78 | (11) | −3 | −41 | ns | ns | ns |
| Vertebra | BV/TV (%) | 12.1 | (2.3) | 11.6 | (2.0) | 12.6 | (1.3) | 11.9 | (1.8) | −4 | −6 | ns | ns | ns |
| | Tb.N. (1/mm) | 3.44 | (0.46) | 3.28 | (0.43) | 3.45 | (0.21) | 3.22 | (0.37) | −5 | −7 | ns | ns | ns |
| | Tb.Sp. (μm) | 149 | (9) | 150 | (13) | 151 | (9) | 156 | (7) | 1 | 4 | ns | ns | ns |
| | Tb.Th. (μm) | 34.9 | (2.2) | 35.3 | (1.9) | 36.8 | (2.0) | 37.0 | (2.1) | 1 | 0 | ns | 0.01 | ns |
| | BS/BV | 116 | (14) | 117 | (10) | 107 | (10) | 108 | (10) | 0 | 2 | ns | 0.014 | ns |
| | Tb.Pf (1/mm) | 31.0 | (5.8) | 34.4 | (3.1) | 29.9 | (2.7) | 33.1 | (4.1) | 11 | 11 | 0.016 | ns | ns |

For abbreviations: see Table 1.
Data are shown as mean (±SD) and percent change in tg vs. WT mice. Results of a full-factorial 2-way ANOVA as main effects of genotype and gender are presented. No influence of gender on genotype effect (gender × genotype interaction) was observed in ANOVA.

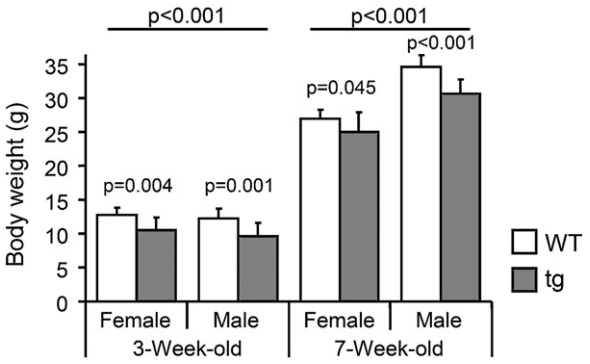


**Fig. 4.** Body weight. Data are shown as means ± SD. Overall *p*-values are for genotype effects by 2-way ANOVA with gender and genotype as factors. Contrast analysis was done for comparisons of tg and WT animals separately for females and males. WT — wild type, tg — transgenic.

*Bone structure and mechanical strength in sexually immature, 3-week-old mice*

Similar to 7-week-old Col2.3-11βHSD2 tg mice, the tibial trabecular bone volume fraction (BV/TV; $p = 0.018$) and trabecular number ($p = 0.02$) were significantly reduced, while trabecular separation (Tb.Sp) was significantly increased ($p = 0.002$) in sexually immature Col2.3-11βHSD2 tg vs. WT mice (Table 2, Fig. 1). However, trabecular thickness and pattern factor were not different between 3-week-old tg and WT animals (Table 2). In Col2.3-11βHSD2 tg mice, tibial cortical bone area (Fig. 2), periosteal and endosteal perimeter and tibia length were significantly decreased ($p<0.01$ each), but cortical thickness remained unchanged when compared to WT animals (Table 2). There was a non-significant trend for lower tibial bone strength in 3-week-old Col2.3-11βHSD2 tg mice when compared to WT littermates (Table 2, Fig. 3). Unlike 7-week-old Col2.3-11βHSD2 tg mice, 3-week-old Col2.3-11βHSD2 tg mice showed similar vertebral trabecular bone structure compared to their WT littermates, with the exception of a significantly increased trabecular pattern factor (i.e. lower connectivity) in the vertebra of Col2.3-11βHSD2 tg mice (Table 2, Fig. 1).

Tibia cortical bone area, periosteal perimeter and cortical thickness were significantly lower in female than in male mice, while most trabecular parameters (for both tibia and vertebra), tibia length and mechanical bone strength revealed no gender-specific differences in 3-week-old mice (Table 2). No interaction between gender and genotype was found for any of the analysed parameters using 2-way ANOVA, indicating that the effect of GC signalling on bone structure in sexually immature mice is similar in both genders.

*Body weight differs significantly between WT and Col2.3-11βHSD2 tg mice*

In both 3 and 7-week-old mice, body weight was significantly lower in Col2.3-11βHSD2 tg animals as compared to their WT littermates ($p<0.001$, each) (Fig. 4, Tables 1, 2). As most of the structural parameters analysed significantly correlate with body weight (data not shown), data were re-analysed using ANCOVA with body weight as covariate (Tables 3, 4). In 7-week-old mice, correction for body weight attenuated the difference between WT and tg animals for most of the parameters measured but the effect of disrupted GC signalling remained significant for cortical and trabecular bone, and for bone strength and stiffness (Table 3). In contrast, adjustment for body weight in 3-week-old mice completely removed all the observed effects in tibial bone parameters (Table 4). Again, no interaction between gender and genotype was found for any of the analysed parameters and these results indicate that the effect of disrupted GC signalling on bone structure remains independent of sex after body weight correction.

## Discussion

The Col2.3-11βHSD2 tg mouse model is characterised by a disruption of intracellular glucocorticoid signalling in mature osteoblasts and osteocytes [10,13,14,17]. As osteoblasts and osteocytes are the key cells regulating local bone metabolism [18,19] this model allows to closely study the role of endogenous glucocorticoids in bone development and maintenance.

**Table 3**
Structural and mechanical bone parameters in 7-week-old mice adjusted for body weight.

| | | 7-week-old mice EMM (SEM) | | | | | | | | 2-way ANCOVA *p*-values | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Female | | | | Male | | | | % change tg vs. WT | | Main effects | | Interaction |
| | | WT | | tg | | WT | | tg | | F | M | Genotype | Gender | Gender × genotype |
| Tibia | BV/TV (%) | 14.0 | (0.9) | 11.9 | (1.1) | 14.2 | (1.3) | 11.4 | (0.8) | −15 | −20 | 0.014 | ns | ns |
| | Tb.N. (1/mm) | 2.60 | (0.17) | 2.33 | (0.22) | 2.81 | (0.25) | 2.36 | (0.16) | −11 | −16 | ns | ns | ns |
| | Tb.Sp. (um) | 218 | (12) | 254 | (16) | 203 | (18) | 236 | (11) | 16 | 16 | 0.015 | ns | ns |
| | Tb.Th. (um) | 53.6 | (0.6) | 51.1 | (0.8) | 50.6 | (0.9) | 48.5 | (0.6) | −5 | −4 | 0.002 | 0.010 | ns |
| | BS/BV | 67 | (1) | 72 | (1) | 75 | (2) | 79 | (1) | 8 | 5 | 0.001 | 0.001 | ns |
| | Tb.Pf (1/mm) | 21.4 | (0.9) | 23.6 | (1.2) | 26.1 | (1.3) | 27.9 | (0.8) | 11 | 7 | ns | 0.007 | ns |
| | Cortical bone area (mm2) | 1.07 | (0.02) | 0.98 | (0.03) | 1.00 | (0.03) | 0.88 | (0.02) | −8 | −12 | <0.001 | 0.035 | ns |
| | Periosteal perimeter (mm) | 6.97 | (0.08) | 6.66 | (0.11) | 6.98 | (0.12) | 6.63 | (0.08) | −4 | −5 | 0.001 | ns | ns |
| | Endosteal perimeter (mm) | 4.57 | (0.12) | 4.32 | (0.16) | 4.90 | (0.18) | 4.34 | (0.11) | −6 | −11 | 0.004 | ns | ns |
| | Cortical thickness (um) | 166 | (5) | 155 | (7) | 152 | (7) | 145 | (5) | −6 | −5 | ns | ns | ns |
| | Tibia length (mm) | 17.5 | (0.1) | 17.4 | (0.1) | 16.8 | (0.2) | 16.8 | (0.1) | 0 | 0 | ns | 0.001 | ns |
| | Maximum load (N) | 13.6 | (0.62) | 10.8 | (0.94) | 13.9 | (0.89) | 9.9 | (0.52) | −20 | −29 | <0.001 | ns | ns |
| | Bending modulus (Stiffness) (MPa) | 656 | (42) | 555 | (64) | 578 | (60) | 395 | (35) | −15 | −32 | 0.007 | ns | ns |
| Vertebra | BV/TV (%) | 22.3 | (1.1) | 19.5 | (1.3) | 18.3 | (1.5) | 15.2 | (1.0) | −13 | −17 | 0.013 | 0.025 | ns |
| | Tb.N. (1/mm) | 4.09 | (0.16) | 3.60 | (0.2) | 3.67 | (0.23) | 3.13 | (0.14) | −12 | −15 | 0.004 | ns | ns |
| | Tb.Sp. (μm) | 192 | (5) | 210 | (6) | 180 | (7) | 202 | (4) | 9 | 12 | 0.001 | ns | ns |
| | Tb.Th. (μm) | 54.3 | (1.0) | 53.8 | (1.3) | 50.2 | (1.5) | 48.7 | (0.9) | −1 | −3 | ns | 0.010 | ns |
| | BS/BV | 66 | (2) | 68 | (3) | 74 | (3) | 78 | (2) | 4 | 4 | ns | 0.013 | ns |
| | Tb.Pf (1/mm) | 9.3 | (1.2) | 12.6 | (1.5) | 15.0 | (1.7) | 18.7 | (1.1) | 35 | 24 | 0.009 | 0.004 | ns |

For abbreviations: see Table 1.
Data are shown as estimated marginal means (EMM) (± SEM) and percent change in tg vs. WT mice. Results of a full-factorial 2-way ANCOVA as main effects of genotype and gender are presented. EMM were calculated with body weight evaluated at 29.3 g for structural parameters and 29.5 g for mechanical parameters. No influence of gender on genotype effect (gender × genotype interaction) was observed in ANCOVA.

**Table 4**
Structural and mechanical bone parameters in 3-week-old mice adjusted for body weight.

| | | 3-week-old mice | | | | | | | | 2-way ANCOVA | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | EMM (SEM) | | | | | | | | *p*-values | | | | |
| | | Female | | | | Male | | | | % change tg vs. WT | | Main effects | | Interaction |
| | | WT | | tg | | WT | | tg | | F | M | Genotype | Gender | Gender × genotype |
| Tibia | BV/TV (%) | 10.0 | (0.9) | 8.7 | (0.8) | 9.2 | (0.8) | 9.6 | (0.9) | −13 | 5 | ns | ns | ns |
| | Tb.N. (1/mm) | 2.79 | (0.21) | 2.56 | (0.2) | 2.62 | (0.2) | 2.74 | (0.22) | −8 | 5 | ns | ns | ns |
| | Tb.Sp. (μm) | 190 | (10) | 196 | (9) | 208 | (9) | 211 | (10) | 3 | 1 | ns | ns | ns |
| | Tb.Th. (μm) | 35.4 | (0.8) | 33.5 | (0.8) | 35.2 | (0.8) | 34.6 | (0.9) | −5 | −2 | ns | ns | ns |
| | BS/BV | 119 | (6) | 133 | (5) | 122 | (5) | 123 | (6) | 12 | 1 | ns | ns | ns |
| | Tb.Pf (1/mm) | 33.2 | (3.0) | 38.5 | (2.8) | 32.7 | (2.8) | 31.0 | (3.1) | 16 | −5 | ns | ns | ns |
| | Cortical bone area (mm2) | 0.39 | (0.01) | 0.41 | (0.01) | 0.37 | (0.01) | 0.39 | (0.01) | 6 | 6 | ns | 0.019 | ns |
| | Periosteal perimeter (mm) | 4.68 | (0.08) | 4.76 | (0.08) | 4.53 | (0.08) | 4.61 | (0.08) | 2 | 2 | ns | ns | ns |
| | Endosteal perimeter (mm) | 3.87 | (0.09) | 3.81 | (0.08) | 3.76 | (0.09) | 3.70 | (0.09) | −2 | −1 | ns | ns | ns |
| | Cortical thickness (um) | 101 | (4) | 115 | (4) | 93 | (4) | 107 | (4) | 14 | 14 | 0.004 | 0.029 | ns |
| | Tibia length (mm) | 12.2 | (0.1) | 12.4 | (0.1) | 12.0 | (0.1) | 12.3 | (0.1) | 2 | 2 | ns | ns | ns |
| | Maximum load (N) | 4.3 | (0.36) | 4.1 | (0.41) | 3.5 | (0.41) | 3.7 | (0.43) | −4 | 5 | ns | ns | ns |
| | Bending modulus (Stiffness) (MPa) | 123 | (15) | 143 | (17) | 88 | (18) | 93 | (18) | 16 | 6 | ns | 0.036 | ns |
| Vertebra | BV/TV (%) | 11.4 | (0.6) | 12.0 | (0.6) | 12.2 | (0.6) | 12.7 | (0.6) | 5 | 4 | ns | ns | ns |
| | Tb.N. (1/mm) | 3.308 | (0.12) | 3.351 | (0.12) | 3.364 | (0.12) | 3.367 | (0.13) | 1 | 0 | ns | ns | ns |
| | Tb.Sp. (μm) | 152 | (3) | 149 | (3) | 153 | (3) | 153 | (3) | −2 | 0 | ns | ns | ns |
| | Tb.Th. (μm) | 34.1 | (0.7) | 35.8 | (0.6) | 36.3 | (0.6) | 37.9 | (0.7) | 5 | 4 | 0.036 | 0.001 | ns |
| | BS/BV | 119 | (4) | 115 | (3) | 109 | (3) | 105 | (4) | −4 | −4 | ns | 0.004 | ns |
| | Tb.Pf (1/mm) | 32.1 | (1.4) | 33.9 | (1.3) | 30.6 | (1.3) | 31.9 | (1.4) | 6 | 4 | ns | ns | ns |

For abbreviations: see Table 1.
Data are shown as estimated marginal means (EMM) (± SEM) and percent change in tg vs. WT mice. Results of a full-factorial 2-way ANCOVA as main effects of genotype and gender are presented. EMM were calculated with body weight evaluated at 11.3 g for structural parameters and 11.7 g for mechanical parameters. No influence of gender on genotype effect (gender × genotype interaction) was observed in ANCOVA.

In a previous study using the same transgenic mouse line, we have shown that endogenous GC signalling in mature osteoblasts is necessary for proper intramembranous bone development in the calvaria [12]. The present study focuses on other skeletal sites, namely the vertebrae and the long bones (tibiae), and includes sexually immature and mature tg and WT mice of both genders. We found that transgenic mice were characterised by lower bone volume, lower trabecular number and higher trabecular separation in tibial trabecular bone, as well as lower tibial cortical bone area and periosteal and endosteal perimeters. These changes resulted in a marked decrease in mechanical bone strength and stiffness in sexually mature, 7-week-old mice. In the tibia, the observed transgene effect was present in 3 and 7-week-old animals, indicating that the biological effect of disrupted GC signalling was independent of sexual maturity. This was not the case for the vertebral bones, where significant differences between tg and WT mice were seen in 7 but not in 3-week-old animals, suggesting that the effects of the transgene at this site may be modulated by age and/or changes in circulating sex hormone levels. Taken together, our results demonstrate that endogenous glucocorticoids are required for normal bone growth but that their effect on bone structure and strength clearly varies by skeletal site and sexual maturity.

In this context, it is important to note that GC can have different and quite opposing effects on bone. While endogenous GC at physiological levels appear to support normal bone development and maintenance, exogenous GC at pharmacological doses cause bone loss leading to osteoporosis. These observations are consistent with previous studies showing that pharmacological concentrations of dexamethasone (e.g. $10^{-6}$ M) are inhibitory to osteoblasts whereas physiological concentrations (e.g. $10^{-8}$ M) stimulate osteoblast differentiation and function [13,20].

It seems noteworthy that the effect of disrupted osteoblastic GC signalling on bone structure was independent of *gender*. This was found to be true at each skeletal site and at each age, even though there were large (and expected) differences in bone parameters between female and male mice, especially in sexually mature animals. There are two possible explanations for this lack of gender-related effects: firstly, endogenous glucocorticoid signalling may interact with bone metabolic pathways that are common to both genders. Secondly, gender-specific, i.e. sex hormone dependent pathways may be influenced to the same extent by endogenous glucocorticoids. Our in vitro studies, using cells derived from Col2.3-11βHSD2 tg and WT animals, support the first possibility as osteoblast-specific disruption of GC signalling was shown to inhibit lineage commitment of mesenchymal progenitor cells through Wnt signalling, in the absence of added sex hormones [13].

Sher et al. [10,11], using the same Col2.3-11βHSD2 tg mouse model, have previously studied the effects of osteoblast-specific GC signalling in 7-week-old and 6-month-old mice. While these studies generally support a role of endogenous glucocorticoids for bone in sexually mature mice, their results differed in a number of important aspects from our study. Thus, the transgene effect on trabecular bone structure was only significant in the vertebrae of female mice, but absent in the vertebrae of males and in the femora of either gender. These results suggested site and gender-specific effects of the transgene in sexually mature mice. In contrast, our study finds no gender effect at all, and any site-specific effects of the transgene were restricted to sexually immature, 3-week-old mice. One possible explanation for these discrepancies may be a difference in statistical methods and/or power. Sher et al. reported a trend for BV/TV to be lower in the femora of tg female mice and in the vertebrae and femora in tg males relative to WT mice. As the number of male animals in that study was significantly smaller than that of female mice, the lack of statistical significance may have been a result of insufficient power to detect a transgene effect in males. Furthermore, some of the variation between these studies may be due to the outbred genetic background of the Col2.3-11βHSD2 transgenic mice and their WT littermates, which could influence the transgene effect [21].

As with all transgenic models, there is always the possibility that the effects observed in transgenic mice result from the insertion of the transgene rather than the transgene activity itself. Kream and co-workers initially established three founder lines termed 516, 515 and 529. These lines showed various levels of transgene expression, with 516 demonstrating strongest and lines 515 and 529 weaker transgene expression. For this reason, only line 516 has been fully characterised

[10,11] and used in further studies [12,13]. However, Kream and co-workers have also analysed bone structural parameters in the 515 transgenic line and found these to be similar to the changes seen in the line studied in our current paper [10, 11, personal communication]. Hence, the effects observed in our and the studies of others are unlikely to be related solely to transgene insertion.

Similar to the observations of Sher et al. [10], we found that Col2.3-11βHSD2 tg mice have a slightly lower body weight than their WT littermates. As body weight can affect skeletal phenotypes, it is possible that the changes in bone structure seen in the tg animals simply resulted from their lower body weight. This possibility has not been addressed in previous studies. We therefore assessed the transgene effects on bone with body weight as a covariate. In 3-week-old mice, correction for body weight removed the observed effects in tibial bone parameters, suggesting that differences in body weight may explain most of skeletal changes seen in sexually immature mice. However, this was not the case in sexually mature, 7-week-old mice where the transgene effect remained significant even after adjustment for body weight. This difference between mature and immature mice suggests a GC-dependent pathway that comes into effect with sexual maturation and, as the observed transgene effect was independent of gender, is common to both genders. One possible mechanism which fits this characteristic is the peripheral conversion of androgen precursors to estrogens by aromatase. This enzyme can be a potent source of locally active estrogens in peripheral tissues and its high expression was shown in the bone tissue of both genders [22]. Glucocorticoids were shown to increase aromatase expression in human osteoblasts in vitro [23,24].

In another transgenic model of cell-specific 11β-HSD2 over-expression, enzyme expression was driven by the osteocalcin gene 2 (OG2) promoter in mature osteoblasts and osteocytes [25]. However, unlike the Col2.3-11βHSD2 model, the OG2-HSD2 transgene had no apparent effect on bone phenotype. Of note, the rat Col2.3 promoter is expressed earlier in osteoblast differentiation while the OG2 promoter becomes active at a later stage in the osteoblast's life cycle [26]. Hence, the differences between the two models are interesting as they indicate that the influence of endogenous GC on bone growth and development may be confined to a narrow window during osteoblast differentiation.

Taken together, the current study provides new insights into the effects of endogenous GC on bone remodeling and strength in sexually immature mice, and how these influence change with aging and sexual maturation. Our results are different from previous studies [10,11,25] in that we have found the transgene effect to be same for males and females (irrespective of sexual maturity or skeletal site) and that site-specific transgene effects are more pronounced in sexually immature then mature mice. We conclude that endogenous glucocorticoid signalling in osteoblasts may play an important role in the development and maintenance of the normal bone phenotype in mice, and that these effects are modulated by sexual maturity and skeletal site, but not by gender.

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