

PAPP-A2 expression by osteoblasts is required for normal postnatal growth in mice

Neilab Amiri and Julian K. Christians

Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby,
BC V5A 1S6

Corresponding author:

Julian Christians

Department of Biological Sciences

Simon Fraser University

8888 University Drive, Burnaby, BC V5A 1S6

Phone: 778-782-5619

e-mail: jkchrist@sfu.ca

Short title: Osteoblast-produced PAPP-A2 is needed for growth

Abstract

Objective

Pregnancy associated plasma protein-A2 (PAPP-A2) is a protease that cleaves insulin-like growth factor binding protein-5 (IGFBP-5), the most abundant IGFBP in bone. Deletion of *Pappa2* reduces postnatal growth and bone length in mice. The aim of this study was to determine whether locally produced PAPP-A2 is required for normal bone growth.

Design

We deleted *Pappa2* primarily in osteoblasts by crossing conditional *Pappa2* deletion mice with mice expressing *Cre* recombinase under the control of the *Sp7* (*Osterix*) promoter. Effects of disrupting *Pappa2* in *Sp7*-expressing cells were examined by measuring body mass and tail length at 3, 6, 10 and 12 weeks of age and bone dimensions at 12 weeks.

Results

Body mass, tail length, and linear bone dimensions were significantly reduced at all ages by osteoblast-specific *Pappa2* deletion. Mice homozygous for the conditional *Pappa2* deletion allele and carrying the *Cre* transgene were smaller than controls carrying the *Cre* transgene, whereas mice homozygous for the conditional *Pappa2* deletion allele were not smaller than controls when comparing mice not carrying the transgene. This result unambiguously demonstrates that PAPP-A2 produced by *Sp7* expressing cells is required for normal growth. However, constitutive *Pappa2* deletion had greater effects than osteoblast-specific *Pappa2* deletion for many traits, indicating that post-natal growth is also affected by other sources of PAPP-A2.

Immunohistochemistry revealed that PAPP-A2 localized in the epiphysis and metaphysis as well as osteoblasts, consistent with a role in bone growth.

Conclusion

Locally-produced PAPP-A2 is required for normal bone growth.

Keywords: Pappalysin-2; Insulin-like growth factor; Insulin-like growth factor binding protein; IGF-axis; bone

Abbreviations

IGF: insulin-like growth factor

IGFBP: insulin-like growth factor binding protein

PAPP-A2: pregnancy associated plasma protein -A2

Introduction

Insulin-like growth factors (IGFs) influence the proliferation, differentiation, and apoptosis of osteoblasts and are required for bone development, mineral deposition, and skeletal growth [1-3]. The availability of IGFs is modulated by IGF binding proteins (IGFBPs) [4], and IGFBP-5 is the most abundant IGFBP in bone, although it acts through IGF-independent as well as IGF-dependent pathways [4, 5].

The IGFBPs are themselves regulated by proteases [1, 6], such as pregnancy associated plasma protein-A2 (PAPP-A2), which is known to cleave IGFBP-5 and potentially IGFBP-3 [7]. Deletion of *Pappa2* would be expected to increase levels of intact IGFBP-5 and therefore reduce IGF availability. Indeed, *Pappa2* deletion mice exhibit reduced postnatal growth [8], with bone lengths reduced more than would be expected given the reduction in body mass alone [9]. Additionally, natural variation in the *Pappa2* gene contributes to variation in skeletal growth in mice [9, 10]. While increased levels of intact IGFBP-5 are a plausible explanation for the phenotypic effects of *Pappa2* deletion, PAPP-A2 may also act through other pathways [11].

While the deletion studies indicate that PAPP-A2 plays a role in postnatal growth, it is not known whether PAPP-A2 acts in a local or systemic manner. Are effects of PAPP-A2 on bone due to locally produced PAPP-A2, and/or PAPP-A2 produced elsewhere? We hypothesized that bone-derived PAPP-A2 plays a role in postnatal skeletal development. Since both IGFs, both IGF receptors, and IGFBP-5 are expressed in osteoblasts [12], we deleted *Pappa2* in osteoblasts to determine whether PAPP-A2 has primarily local or systemic effects. This was achieved by crossing conditional *Pappa2* deletion mice with mice expressing *Cre* recombinase under the control of the *Osterix/Sp7* (*Osx/Sp7*) promoter [13]. Furthermore, we sought to characterize PAPP-A2 expression in the long bones.

Materials and methods

Ethics Statement

All work was carried out in accordance with the guidelines of the Canadian Council on Animal Care and was approved by the SFU University Animal Care Committee (protocol 1035B-11).

***Pappa2* deletion mice**

Conditional *Pappa2* deletion mice with a C57BL/6 background were generated as previously described [9], such that mouse exon 2 (homologous to human exon 3) and a PGK-Neo selection cassette were flanked by LoxP sites (“floxed”). The selection cassette was flanked by FRT sequences and was removed by breeding with mice carrying a *Flp* recombinase transgene (Jackson Laboratory stock number 011065). Following *Flp* recombinase mediated removal of the selection cassette, the *Flp* recombinase transgene was removed by further breeding to produce mice with a floxed exon 2 (*Pappa2^{fl}*) and no selection cassette or *Flp* transgene. Thus, none of the mice in this study harboured the selection cassette.

Deletion of exon 2 in *Sp7*-expressing cells was achieved by crossing conditional deletion mice to mice expressing *Cre* recombinase under the control of the *Osterix/Sp7* (*Osx/Sp7*) promoter (hereafter referred to as *Sp7-Cre*; Jackson Laboratory stock number 006361). The expression of this transgene is not confined to osteoblasts, and has been detected in the brain, intestinal epithelium, and olfactory cells [14, 15]. However, it is widely used for conditional deletion in the osteoblast lineage and so we refer to the deletion as “osteoblast-specific” for brevity. *Cre*-mediated deletion of exon 2 and splicing of exon 1 to exon 3 is expected to produce an early stop codon, and we have previously shown that this results in PAPP-A2 protein being undetectable in the placenta, despite being abundant in wild-type mice [9].

To determine the effect of osteoblast-specific *Pappa2* deletion, mice heterozygous for the conditional *Pappa2* allele and hemizygous for the *Sp7-Cre* transgene (*Pappa2^{wt/fl}*; *Sp7-Cre*) were paired with mice homozygous for the conditional *Pappa2* allele with no transgene (*Pappa2^{fl/fl}*) to produce litters in which four genotypes were present: homozygous or heterozygous for the conditional *Pappa2^{fl}* allele and with or without the *Sp7-Cre* transgene. Our previous work suggested that the effects of constitutive *Pappa2* deletion on growth are recessive [9], therefore we expected to detect effects of osteoblast-specific deletion by comparing homozygotes (*Pappa2^{fl/fl}*) and heterozygotes (*Pappa2^{wt/fl}*), but only among mice carrying the *Sp7-Cre* transgene. These offspring were used for measurement of postnatal growth (described below). Removal of *Pappa2* exon 2 in bone was determined by PCR genotyping (described below). Postnatal growth was measured in 46 males and 39 females, with sample sizes for each genotype as follows: *Pappa2^{wt/fl}*: 25 males and 8 females; *Pappa2^{fl/fl}*: 8 males and 12 females; *Pappa2^{wt/fl}*; *Sp7-Cre*: 8 males and 13 females; *Pappa2^{fl/fl}*; *Sp7-Cre*: 5 males and 6 females.

Constitutive *Pappa2* deletion mice

Constitutive PAPP-A2 deletion mice were described previously and the data from this earlier study [9] were used to compare the effects of whole-body *Pappa2* deletion with the effects of osteoblast-specific *Pappa2* deletion (this study). Briefly, conditional deletion mice were crossed to mice expressing *Cre* recombinase under the control of a human cytomegalovirus minimal promoter (Jackson Laboratory stock number 006054). Mice heterozygous for the constitutive *Pappa2* disruption were then paired to produce litters in which all three genotypes were present resulting in 40 male and 35 female offspring, with sample sizes for each genotype as follows: *Pappa2^{wt/wt}*: 7 males and 7 females; *Pappa2^{wt/KO}*: 23 males and 18 females; *Pappa2^{KO/KO}*: 10 males and 10 females. The constitutive and osteoblast-specific deletion mice derive from the

same line of conditional deletion mice. This line and all of the other transgenic lines used in these experiments have a C57BL/6 background. All mice were housed in the same facility under the same conditions.

Genotyping

Mice were ear-clipped at three weeks of age and DNA extraction was performed by standard methods. PCR genotyping was used for the determination of (a) *Pappa2* alleles (*Pappa2*^{wt}, *Pappa2*^{fl}, and *Pappa2*^{KO}), and (b) the presence/absence of the *Sp7-Cre* transgene. Genotyping of *Pappa2* alleles used three primers designed to yield bands of different sizes for the three alleles (166 bp for *Pappa2*^{wt}, 305 bp for *Pappa2*^{fl} and 272 bp for *Pappa2*^{KO}). Primer sequences are as follows: KO_prox (5'-CAG CAA AGG AAA TTT GTG CT-3'), KO_exon2 (5'-GGT CAA ATG AAA CTT CCC TCC-3'), KO_dist2 (5'-CTC TTG CAT GCC TCC ACT AC-3'). The genotyping reactions for *Sp7-Cre* included two primer pairs recommended by the Jackson Laboratory: one to amplify a fragment from the *Sp7-Cre* transgene and another to amplify a positive control fragment to confirm that the PCR was successful. The positive control primers target an exon of the *Interleukin 2* gene on chromosome 3. Primer sequences are as follows: Cre_A (5'-GCGGTCTGGCAGTAAAACTATC-3'), Cre_B (5'-GTGAAACAGCATTGCTGTCACTT-3'), Cre_+ve_A (5'-CTAGGCCACAGAATTGAAAGATCT-3'), Cre_+ve_B (5'-GTAGGTGGAAATTCTAGCATCATCC-3').

Phenotypes

Body mass and tail length were measured at 3, 6, 10, and 12 weeks of age in offspring from the cross between *Pappa2*^{wt/fl}; *Sp7-Cre* and *Pappa2*^{fl/fl} mice. Mice were sacrificed at 12 weeks of age and frozen at -20°C. These mice were thawed at a later date, the skin and internal organs were

removed and the carcasses were dried to a constant weight before being exposed to dermestid beetles for removal of soft tissue, allowing the following bone measurements: mandible length (distance from the tip of the angular process to the anterior edge of the molars), mandible height (from the coronoid process to the tip of the angular process), and the lengths of the skull, humerus, ulna/radius, femur, tibia, and pelvic girdle. Where applicable, we measured bones from both sides and calculated the mean. All skeletal dimensions were measured with digital callipers (± 0.01 mm) and measurements were performed in triplicate. To confirm bone-specific *Pappa2* disruption in *Pappa2^{fl/fl}*; *Sp7-Cre* mice, four mice were sacrificed at 6 weeks of age to collect tissues for genotyping. Samples of ear, bone, heart, liver, lung, kidney, spleen, and muscle were collected and stored at -80°C . DNA was extracted using the DNeasy Blood & Tissue Kit (Cat. No. 69504) from Qiagen (Hilden). PCR was performed to determine the presence or absence of the 272 bp deletion allele in these samples. Standard PCR, rather than quantitative PCR, was used since deletion was only expected in a small subset of cells.

Measurement of circulating IGFBP-5 levels

Since IGFBP-5 is the only confirmed target of PAPP-A2, we assessed whether osteoblast-specific *Pappa2* deletion affected plasma levels of IGFBP-5 in 10 juvenile (18-19 day old) mice, measured by ELISA (DY578; R&D Systems).

Immunohistochemistry

Long bones from juvenile (19 days) mice were dissected in cold PBS. All soft tissues were carefully removed and samples fixed in 10% neutral buffered formalin for 72 hours. Femorotibial joints were subsequently decalcified in 10% EDTA and embedded in paraffin. Sections were deparaffinised and rehydrated according to standard protocols and heat-antigen retrieval was performed in 10mM citrate buffer pH 6.0. Immunohistochemistry was performed using HRP-

AEC (CTS009; R&D Systems). Sections were incubated overnight at 4°C with 10 µg/mL of polyclonal goat-anti-human PAPP-A2 antibody (AF1668; R&D Systems) or 5 µg/mL of polyclonal goat-anti mouse IGFBP-5 (AF578; R&D Systems) or matched concentrations of normal goat IgG control (AB-108-C; R&D Systems) as a negative control. Previously, we have shown by Western blotting that this PAPP-A2 antibody reacts with a protein of the expected size in mouse placenta (which is known to express abundant PAPP-A2) but not with other adult mouse tissues [16], suggesting that it is specific for the mouse ortholog of human PAPP-A2. Sections were counterstained using Gills No. 3 Hematoxylin diluted 1:5 in sterile water. The intensity of PAPP-A2 staining in osteoblasts of 7 juvenile mice (3 *Pappa2^{fl/fl}* and 4 *Pappa2^{fl/fl}; Sp7-Cre*) was scored by two individuals blind to genotype.

Statistical Analyses

All statistical analyses were performed using general linear models (proc GLM) and repeated measures analyses (proc MIXED) in SAS, Version 9.3 (SAS Institute Inc., Cary, NC).

To compare the effects of osteoblast -specific and constitutive *Pappa2* deletion, we combined the phenotypic data from the present study with that from our previous study of constitutive *Pappa2* deletion [9]. Phenotypes were analysed using a general linear model including the effects of experiment (osteoblast -specific or constitutive deletion), genotype (*Pappa2^{wt/fl}*, *Pappa2^{fl/fl}*, *Pappa2^{wt/fl};Sp7-Cre*, *Pappa2^{fl/fl};Sp7-Cre*, *Pappa2^{wt/wt}*, *Pappa2^{wt/KO}*, *Pappa2^{KO/KO}*, nested within experiment), litter identity (nested within experiment), and sex. In this analysis, we wished to test whether the effect of osteoblast -specific deletion (*Pappa2^{wt/fl};Sp7-Cre* vs. *Pappa2^{fl/fl};Sp7-Cre*) was different than the effect of constitutive deletion (*Pappa2^{wt/KO}* vs. *Pappa2^{KO/KO}*; we compared heterozygotes to homozygotes because this was the comparison in the osteoblast-specific experiment). Differences between genotypes were

estimated using ESTIMATE statements, and the difference between estimates was tested using a CONTRAST statement in proc GLM. Prior to this analysis, we standardized all phenotypes within each experiment by subtracting the mean and dividing by the standard deviation of the *Pappa2*^{wt/fl}; *Sp7-Cre* mice (osteoblast-specific experiment) or the *Pappa2*^{wt/KO} mice (constitutive experiment). As a result, the *Pappa2*^{wt/fl}; *Sp7-Cre* mice and the *Pappa2*^{wt/KO} mice had a mean of 0 and a standard deviation of 1 for every phenotype, and the phenotypes of all mice were expressed in standard deviation units. Expressing the data as standard deviation units allows comparison of the magnitude of effects between traits.

Results

***Pappa2* expression and deletion in bone**

In the femur and tibia, immunostaining for PAPP-A2 and its target, IGFBP-5, was present in the epiphysis and metaphysis, including the osteoblasts (Fig. 1). We confirmed the presence of the deleted *Pappa2* allele in bone using PCR. In *Pappa2*^{fl/fl}; *Sp7-Cre* mice (i.e., those carrying the *Sp7-Cre* transgene), PCR of DNA from ear-clip, liver, lung, heart, muscle, kidney, spleen and bone yielded the deletion band only in bone samples, although a faint band was present in lung from one of the mice (Fig. 2). Blind scoring of immunohistochemistry images revealed a trend of reduced/absent staining in osteoblasts of *Pappa2*^{fl/fl}; *Sp7-Cre* mice (Fig. 3), although *Sp7-Cre*-mediated deletion did not appear to be complete.

Plasma IGFBP-5

We measured plasma IGFBP-5 to determine whether osteoblast-specific deletion affected the circulating levels of the target of *Pappa2* among mice carrying the *Sp7-Cre* transgene. In a model including effects of litter and *Pappa2* genotype, there was no difference in IGFBP-5 levels

between *Pappa2* genotypes in 18-19 day old mice ($F_{1,5} = 0.00$, $P = 0.99$; *Pappa2*^{fl/fl}: 60 ± 5 ng/mL, $n = 5$; *Pappa2*^{wt/fl}: 60 ± 4 ng/mL, $n = 5$; values are least square means \pm standard error). Sex was initially included in the model but was not significant.

Effects of osteoblast-specific *Pappa2* deletion

In offspring of *Pappa2*^{wt/fl};*Sp7-Cre* and *Pappa2*^{fl/fl} mice, genotypic ratios deviated from the expected Mendelian values. The numbers of *Pappa2*^{wt/fl}: *Pappa2*^{fl/fl}: *Pappa2*^{wt/fl};*Sp7-Cre*: *Pappa2*^{fl/fl};*Sp7-Cre* mice were 34, 25, 22, 15. The ratio of offspring that inherited the *Sp7-Cre* transgene to those that did not was 37:59, which deviated from the expected 1:1 ratio ($\chi^2_1 = 5.04$, $p = 0.02$), while the ratio of *Pappa2*^{wt/fl} to *Pappa2*^{fl/fl} offspring (56:40) did not differ from expected ($\chi^2_1 = 2.67$, $p = 0.10$). Furthermore, the ratio of mice that inherited the *Sp7-Cre* transgene did not differ between *Pappa2*^{wt/fl} (22:34) and *Pappa2*^{fl/fl} (15:25) mice ($\chi^2_1 = 0.03$, $p = 0.86$), indicating that the deficiency of mice carrying the *Sp7-Cre* transgene was not more severe in one *Pappa2* genotype than in the other. The deficiency of mice carrying the *Sp7-Cre* transgene may reflect an effect of the transgene on survival. However, we observed only one dead pup among these litters suggesting that if there is an effect of the transgene on survival, mortality occurs *in utero* or very soon after birth.

Osteoblast-specific *Pappa2* deletion effects were examined by measuring body mass and tail lengths at 3, 6, 10 and 12 weeks of age and measuring linear bone dimensions after cull at 12 weeks of age. We predicted that postnatal growth would be reduced in *Pappa2*^{fl/fl};*Sp7-Cre* mice compared to *Pappa2*^{wt/fl};*Sp7-Cre* mice due to deletion of *Pappa2* in osteoblasts, but that there would be no difference between *Pappa2*^{fl/fl} and *Pappa2*^{wt/fl} mice not carrying the *Sp7-Cre* transgene. Therefore, we predicted an interaction between the *Pappa2* allele genotype and the *Cre* genotype. Combining measures from all ages using MANOVA, the statistical interaction

between the *Pappa2* allele genotype and the *Sp7-Cre* genotype was significant for body mass (Wilks' Lambda $F_{4,68} = 3.75$; $P = 0.0082$) and tail length (Wilks' Lambda $F_{4,69} = 3.34$; $P = 0.0148$). Similarly, the interaction was also significant in the repeated measures analysis of body mass ($F_{1,72}=12.78$; $P=0.0006$) and tail length ($F_{1,72}=6.23$; $P=0.0148$). To determine the nature of the interaction, we used the CONTRAST statement in proc GLM to compare the least squares means for the *Pappa2*^{fl/fl} and *Pappa2*^{wt/fl} genotypes separately for mice with and without the *Sp7-Cre* transgene. For body mass and tail length at all ages, the differences between *Pappa2* genotypes were significant for mice with the *Sp7-Cre* transgene, and not significant for mice without the *Sp7-Cre* transgene (Table 1 and Fig. 4). The *Sp7-Cre* transgene also reduced body size independent of the *Pappa2* allele genotype (Fig. 4), as previously found [14]. However, the significant statistical interaction demonstrates that, in addition to the effect of the transgene *per se*, there is an effect of osteoblast-specific *Pappa2* deletion: *Pappa2*^{fl/fl} mice are smaller than *Pappa2*^{wt/fl} mice when comparing mice carrying the transgene, but not when comparing mice not carrying the transgene. We found no significant interactions between sex and *Pappa2* and/or *Sp7-Cre* genotype, indicating that the effects of *Pappa2* deletion were not sex-specific.

We observed similar results for linear bone dimensions (Table 1). There was a significant interaction between the *Pappa2* allele genotype and the *Sp7-Cre* genotype for bones of the head (skull and mandible dimensions; MANOVA Wilks' Lambda $F_{3,53} = 8.72$; $P = 0.0001$) and the long bones (humerus, radius/ulna, femur and tibia; MANOVA Wilks' Lambda $F_{4,69} = 3.40$; $P = 0.0135$). As with body mass and tail length, in all bones there was a significant difference between *Pappa2* genotypes in mice with the *Sp7-Cre* transgene, but not in mice without the *Sp7-Cre* transgene (Table 1). Interactions between sex and genotype were initially included in models,

but were not found to be significant for any trait, indicating that the effects of *Pappa2* deletion were not sex-specific.

Comparison of effects of osteoblast-specific and constitutive *Pappa2* deletion

For body mass at 10 weeks, tail length at 3, 6 and 10 weeks, mandible dimensions and the length of the femur, the difference between *Pappa2*^{wt/KO} and *Pappa2*^{KO/KO} mice was significantly greater than the difference between *Pappa2*^{wt/fl}; *Sp7-Cre* and *Pappa2*^{fl/fl}; *Sp7-Cre* mice, indicating that the effect of constitutive PAPP-A2 deletion was greater than the effects of osteoblast-specific deletion (Table 2). For the measurements of all bones apart from the mandible and femur, there was no significant difference between the estimated effects of the constitutive and osteoblast-specific deletion (Table 2).

Discussion

This is the first study to investigate the role of bone-derived PAPP-A2 using *Osterix/Sp7* (*Osx/Sp7*) driven *Cre* recombinase expression, a method previously used for osteoblast-specific gene disruption [13]. We detected the *Pappa2* deletion allele in bone only, although some *Cre* expression in the brain, intestinal epithelium, and olfactory cells has been reported with this transgene [14, 15]. To assess the phenotypic consequences of *Sp7-Cre*-driven *Pappa2* disruption, *Pappa2*^{wt/fl}; *Sp7-Cre* mice were mated with *Pappa2*^{fl/fl} mice lacking *Cre* to produce littermates homozygous or heterozygous for the conditional *Pappa2* allele, with or without the *Sp7-Cre* transgene. We found a significant interaction between the *Pappa2* and *Sp7-Cre* genotypes for all traits, such that there was a difference between *Pappa2* genotypes only when the *Sp7-Cre* transgene was present; homozygous conditional deletion mice were smaller than heterozygous littermates also carrying the transgene. These results demonstrate unambiguously that the

phenotypic effects are due to *Sp7-Cre*-driven *Pappa2* disruption, and not to effects of the *Sp7-Cre* transgene *per se*; *Pappa2* disruption mice are smaller than controls that also carry the *Sp7-Cre* transgene.

Osteoblast-specific disruption of *Pappa2* resulted in a significant decrease in body mass and skeletal size at all ages (Table 1 and Fig. 4), suggesting that osteoblast-produced PAPP-A2 affects postnatal growth of bones and other tissues. The phenotypic difference between constitutive knock-outs and controls (*Pappa2*^{KO/KO} vs. *Pappa2*^{wt/KO}) was significantly larger than that between osteoblast-specific deletion mice and controls (*Pappa2*^{fl/fl}; *Sp7-Cre* vs. *Pappa2*^{wt/fl}; *Sp7-Cre*) for body mass, tail length, mandible dimensions and femur length, indicating that postnatal growth is affected by PAPP-A2 produced by other cell types as well as that produced by *Sp7*-expressing cells. However, blind-scoring of PAPP-A2 staining in osteoblasts of *Pappa2*^{fl/fl}; *Sp7-Cre* mice suggested that disruption was incomplete, perhaps due to variable *Sp7-Cre* expression. Alternatively, since PAPP-A2 is a secreted protein, we cannot rule out the possibility that the PAPP-A2 detected at osteoblasts was produced elsewhere and localized to osteoblasts.

The mechanisms underlying the phenotypic effects of *Pappa2* deletion remain unknown. The circulating levels of IGFBP-5, a target of PAPP-A2, were not higher in *Pappa2*^{fl/fl}; *Sp7-Cre* mice, whereas circulating IGFBP-5 levels are approximately doubled in constitutive PAPP-A2 deletion mice, as expected [17]. While osteoblast-specific *Pappa2* deletion did not affect circulating IGFBP-5 levels, increased local IGFBP-5 levels, leading to reduced IGF availability, is a plausible mechanism. Locally-produced IGF-I influences bone development and physiology [18], and deletion of IGF-I in chondrocytes, osteocytes, or cells of the osteoblastic lineage reduces bone growth [19-21]. Alternatively, a recent study in zebrafish has linked PAPP-A2 to

other pathways [11], raising the possibility that the effects we observed could have been due to IGFBP-5-independent effects. However, PAPP-A2's paralog, PAPP-A, has also been implicated in bone growth [22, 23] and these effects appear to be due to its IGFBP proteolytic activity [24, 25] .

The present study documents PAPP-A2 expression in the long bones and demonstrates that PAPP-A2 produced both by *Sp7*-expressing cells and other cell-types is important for post-natal growth in mice. Whether local PAPP-A2 influences postnatal growth through proteolysis of local IGFBP-5 and increased IGF-I bioavailability, IGF-independent pathways, or even as yet unstudied IGFBP-5 independent pathways will require further study.

Acknowledgements

We thank Diana Duan, Katrina Juwono, James Topham and Kaitlyn Martel for assistance with dissections, bone measurements, genotyping and IHC scoring. We also thank the Animal Care staff at Simon Fraser University for assistance with animal maintenance and blood sampling. Additionally, we are indebted to Dr. Boa Ping Song and the histology department at the Child and Family Research Institute for their services. This work was supported by a NSERC (Canada) Discovery Grant to JKC (grant number 326791-2011).

References

- [1] K.E. Govoni, D.J. Baylink, S. Mohan, The multi-functional role of insulin-like growth factor binding proteins in bone. *Pediatr. Nephrol.* 20 (3) (2005) 261-268.
- [2] S. Mohan, C. Richman, R.Q. Guo, Y. Amaar, L.R. Donahue, J. Wergedal, D.J. Baylink, Insulin-like growth factor regulates peak bone mineral density in mice by both growth hormone-dependent and -independent mechanisms. *Endocrinology* 144 (3) (2003) 929-936.
- [3] W. Zhang, X. Shen, C. Wan, Q. Zhao, L. Zhang, Q. Zhou, L. Deng, Effects of insulin and insulin-like growth factor 1 on osteoblast proliferation and differentiation: Differential signalling via akt and ERK. *Cell Biochem. Funct.* 30 (4) (2012) 297-302.
- [4] S. Mohan, D.J. Baylink, IGF-binding proteins are multifunctional and act via IGF-dependent and -independent mechanisms. *J. Endocrinol.* 175 (1) (2002) 19-31.
- [5] N. Miyakoshi, C. Richman, Y. Kasukawa, T.A. Linkhart, D.J. Baylink, S. Mohan, Evidence that IGF-binding protein-5 functions as a growth factor. *J. Clin. Invest.* 107 (1) (2001) 73-81.
- [6] R.C. Bunn, J.L. Fowlkes, Insulin-like growth factor binding protein proteolysis. *Trends in Endocrinology and Metabolism* 14 (4) (2003) 176-181.
- [7] M.T. Overgaard, H.B. Boldt, L.S. Laursen, L. Sottrup-Jensen, C.A. Conover, C. Oxvig, Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase. *J. Biol. Chem.* 276 (24) (2001) 21849-21853.
- [8] C.A. Conover, H.B. Boldt, L.K. Bale, K.B. Clifton, J.A. Grell, J.R. Mader, E.J. Mason, D.R. Powell, Pregnancy-associated plasma protein-A2 (PAPP-A2): Tissue expression and biological consequences of gene knockout in mice. *Endocrinology* 152 (7) (2011) 2837-2844.

- [9] J.K. Christians, D.R. de Zwaan, S.H.Y. Fung, Pregnancy associated plasma protein A2 (PAPP-A2) affects bone size and shape and contributes to natural variation in postnatal growth in mice. *Plos One* 8 (2) (2013) e56260.
- [10] J.K. Christians, A. Hoeflich, P.D. Keightley, PAPP2, an enzyme that cleaves an insulin-like growth-factor-binding protein, is a candidate gene for a quantitative trait locus affecting body size in mice. *Genetics* 173 (3) (2006) 1547-1553.
- [11] K. Kjaer-Sorensen, D.H. Engholm, M.R. Jepsen, M.G. Morch, K. Weyer, L.L. Hefting, L.L. Skov, L.S. Laursen, C. Oxvig, Papp-a2 modulates development of cranial cartilage and angiogenesis in zebrafish embryos. *J. Cell. Sci.* 127 (23) (2014) 5027-5037.
- [12] E.M. Wang, J. Wang, E. Chin, J.A. Zhou, C.A. Bondy, Cellular-patterns of insulin-like growth-factor system gene-expression in murine chondrogenesis and osteogenesis. *Endocrinology* 136 (6) (1995) 2741-2751.
- [13] S.J. Rodda, A.P. McMahon, Distinct roles for hedgehog and canonical wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 133 (16) (2006) 3231-3244.
- [14] R.A. Davey, M.V. Clarke, S. Sastra, J.P. Skinner, C. Chiang, P.H. Anderson, J.D. Zajac, Decreased body weight in young osterix-cre transgenic mice results in delayed cortical bone expansion and accrual. *Transgenic Res.* 21 (4) (2012) 885-893.
- [15] J. Chen, Y. Shi, J. Regan, K. Karuppaiah, D.M. Ornitz, F. Long, Osx-cre targets multiple cell types besides osteoblast lineage in postnatal mice. *Plos One* 9 (1) (2014) e85161.
- [16] J. Wang, Q. Qiu, M. Haider, M. Bell, A. Gruslin, J.K. Christians, Expression of pregnancy-associated plasma protein A2 during pregnancy in human and mouse. *J. Endocrinol.* 202 (3) (2009) 337-345.

- [17] J.K. Christians, A.K. Bath, N. Amiri, *Pappa2* deletion alters IGFs but has little effect on glucose disposal or adiposity. *Growth Hormone & IGF Research* (2015)
doi:10.1016/j.ghir.2015.07.001.
- [18] S. Yakar, H. Courtland, D. Clemmons, IGF-1 and bone: New discoveries from mouse models. *Journal of Bone and Mineral Research* 25 (12) (2010) 2267-2276.
- [19] M.H.-. Sheng, X. Zhou, L.F. Bonewald, D.J. Baylink, K.-W. Lau, Disruption of the insulin-like growth factor-1 gene in osteocytes impairs developmental bone growth in mice. *Bone* 52 (1) (2013) 133-144.
- [20] K.E. Govoni, S.K. Lee, Y. Chung, R.R. Behringer, J.E. Wergedal, D.J. Baylink, S. Mohan, Disruption of insulin-like growth factor-I expression in type II alpha I collagen-expressing cells reduces bone length and width in mice. *Physiological Genomics* 30 (3) (2007) 354-362.
- [21] K.E. Govoni, J.E. Wergedal, L. Florin, P. Angel, D.J. Baylink, S. Mohan, Conditional deletion of insulin-like growth factor-I in collagen type 1 alpha 2-expressing cells results in postnatal lethality and a dramatic reduction in bone accretion. *Endocrinology* 148 (12) (2007) 5706-5715.
- [22] C.A. Conover, L.K. Bale, M.T. Overgaard, E.W. Johnstone, U.H. Laursen, E.M. Fuchtbauer, C. Oxvig, J. van Deursen, Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development. *Development* 131 (5) (2004) 1187-1194.
- [23] S.J. Tanner, T.E. Hefferan, C.J. Rosen, C.A. Conover, Impact of pregnancy-associated plasma protein-A deletion on the adult murine skeleton. *Journal of Bone and Mineral Research* 23 (5) (2008) 655-662.

[24] X. Qin, J.E. Wergedal, M. Rehage, K. Tran, J. Newton, P. Lam, D.J. Baylink, S. Mohan, Pregnancy-associated plasma protein-A increases osteoblast proliferation in vitro and bone formation in vivo. *Endocrinology* 147 (12) (2006) 5653-5661.

[25] D. Phang, M. Rehage, B. Bonafede, D. Hou, W. Xing, S. Mohan, J.E. Wergedal, X. Qin, Inactivation of insulin-like-growth factors diminished the anabolic effects of pregnancy-associated plasma protein-A (PAPP-A) on bone in mice. *Growth Hormone & IGF Research* 20 (3) (2010) 192-200.

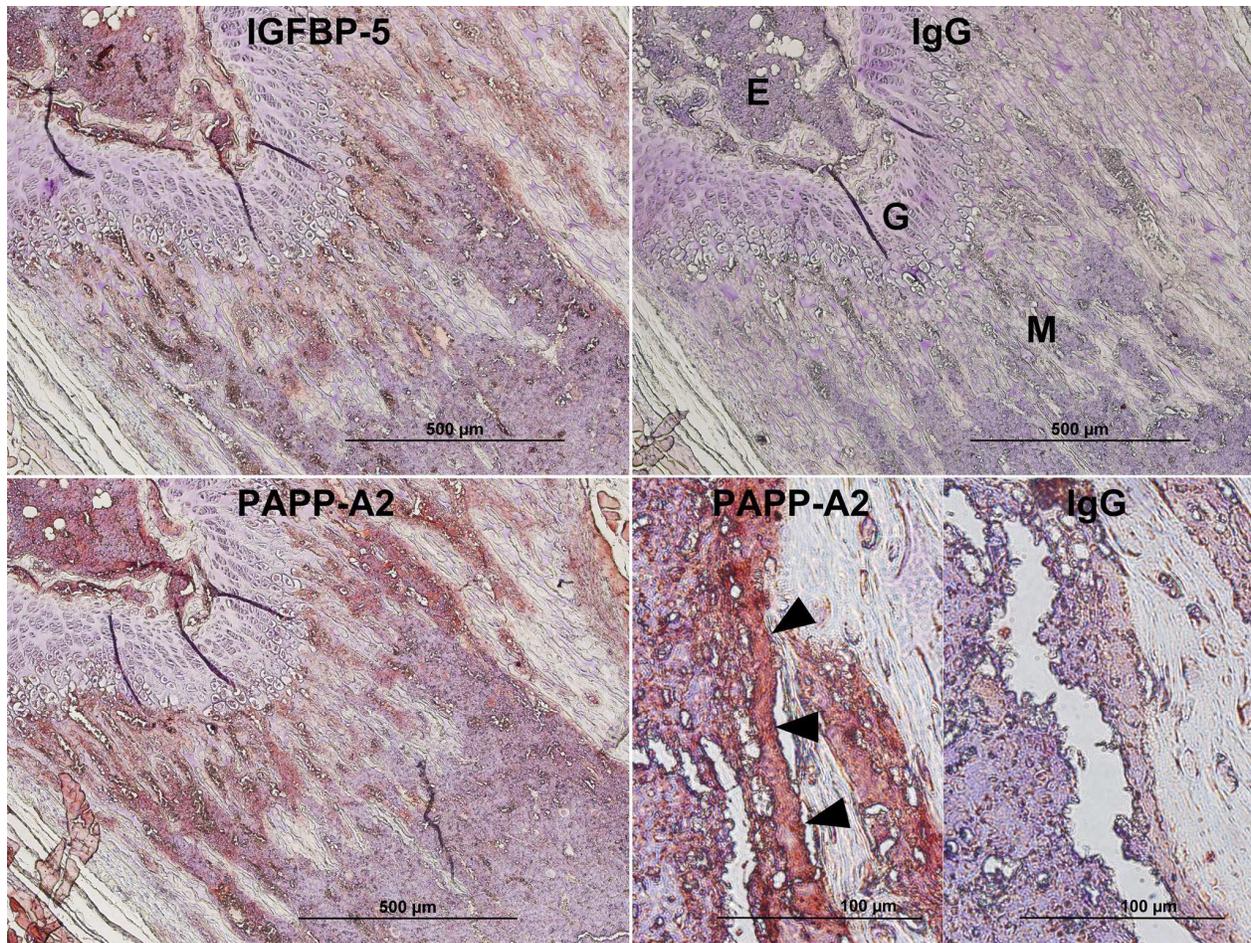


Figure 1. IGFBP-5 and PAPP-A2 localization in long bones. Serial femoral sections stained for IGFBP-5, PAPP-A2 and negative control (IgG), counterstained with hematoxylin. The epiphysis (E), growth plate (G) and metaphysis (M) are labeled in the negative control image. Higher magnification images at bottom right are from the femur of a different mouse, showing staining for PAPP-A2 in osteoblasts (arrowheads).

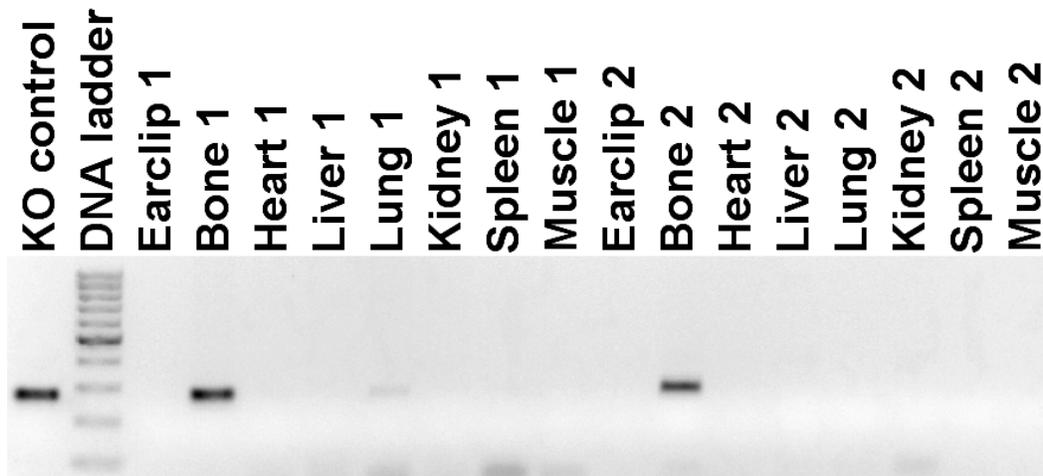


Figure 2. *Pappa2* deletion in bone. PCR amplification of a 272 bp fragment of the *Pappa2* deletion allele (*Pappa2*^{KO}) in various tissues of two *Pappa2*^{fl/fl};*Sp7-Cre* mice (1 and 2). A positive control sample (earclip DNA from a homozygous constitutive deletion mouse) is in lane 1 and a 100 bp DNA ladder is in lane 2.

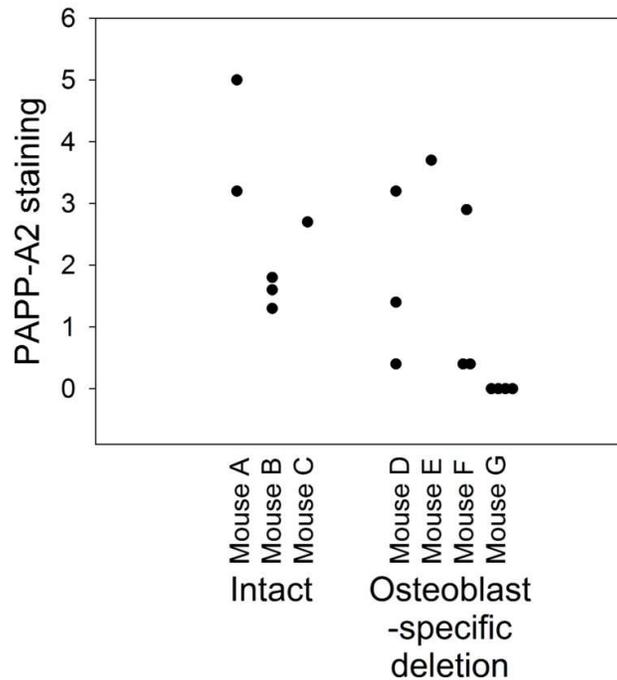
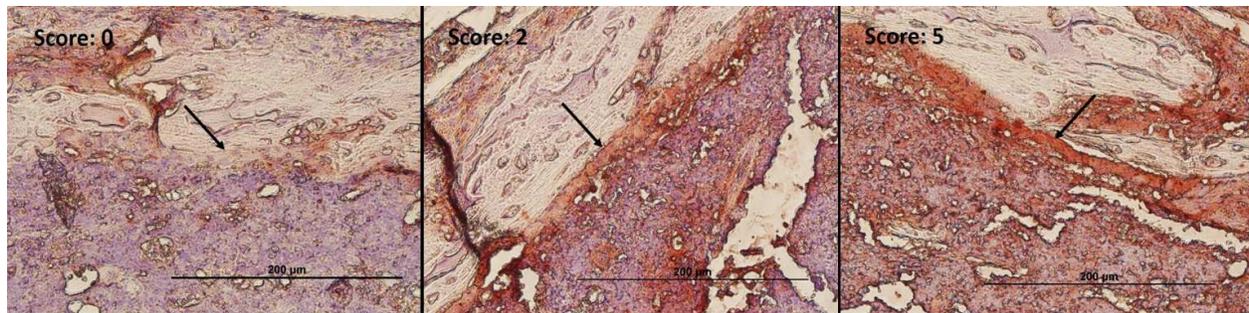


Figure 3. Blind-scoring of PAPP-A2 expression in osteoblasts. Upper panels: Representative scores for PAPP-A2 staining in osteoblasts on a scale of 0 (minimal staining) to 5 (intense staining). Arrow heads indicate continuous layer of osteoblasts at the junction of bone and marrow. Lower panel: Distribution of PAPP-A2 staining scores in osteoblasts of femurs from 3 control (intact) mice and 4 osteoblast-deletion mice. Each point represents the average score for one image from two individuals blind to genotype.

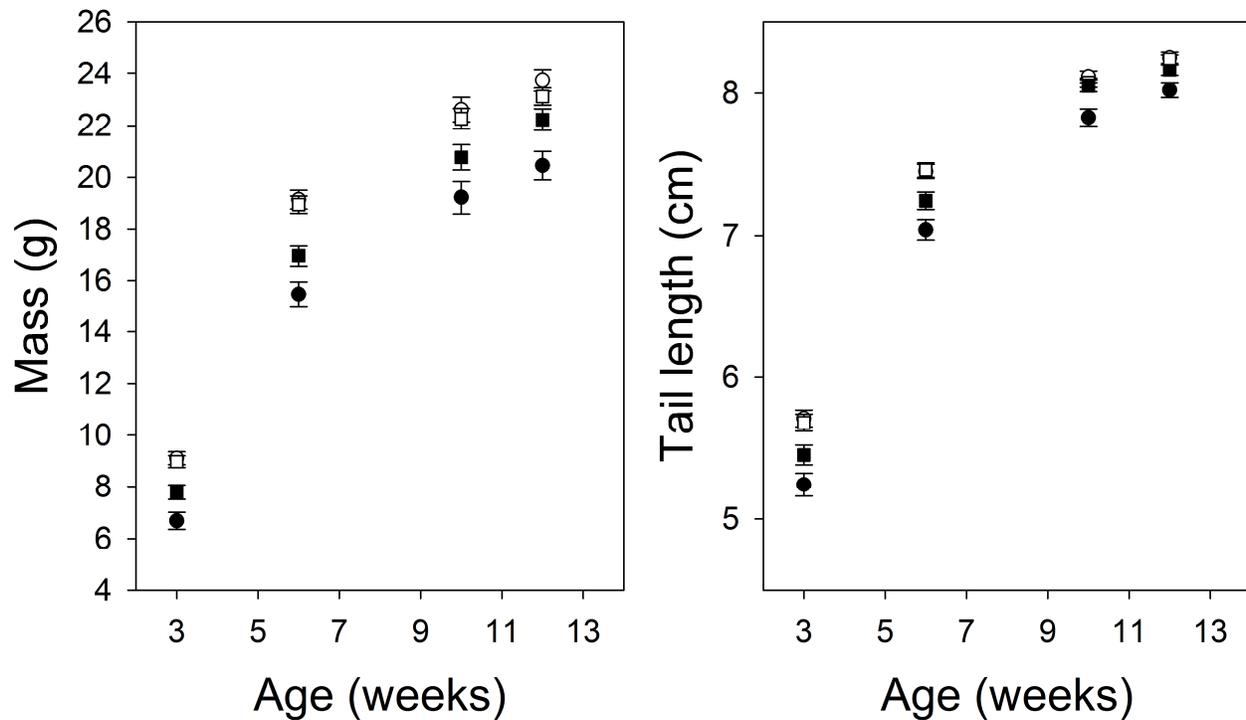


Figure 4. Effects of osteoblast-specific *Pappa2* deletion on post-natal growth. Body mass and tail length at 3, 6, 10 and 12 weeks of age in mice heterozygous (*Pappa2*^{wt/fl}; squares) and homozygous (*Pappa2*^{fl/fl}; circles) for the conditional *Pappa2* deletion allele, with (closed symbols) or without (open symbols) the *Sp7-Cre* transgene. Values are least square means (\pm standard error) from a model including effects of litter, sex, *Pappa2* genotype, *Sp7-Cre* genotype and the interaction between *Pappa2* and *Sp7-Cre* genotype, n = 11-32 per genotype.

Table 1. Effects of osteoblast-specific *Pappa2* deletion on postnatal growth.

Trait	<i>Pappa2^{fl/fl}</i> ; <i>Sp7-Cre⁺¹</i>	<i>Pappa2^{wt/fl}</i> ; <i>Sp7-Cre⁺¹</i>	<i>Pappa2^{fl/fl}</i> ; <i>Sp7-Cre⁻¹</i>	<i>Pappa2^{wt/fl}</i> ; <i>Sp7-Cre⁻¹</i>	P value (<i>Pappa2*Cre</i> interaction)	P value (<i>Cre+</i> contrast)	P value (<i>Cre-</i> contrast)
3 week mass (g)	6.7 ± 0.3	7.8 ± 0.3	9.1 ± 0.3	9.0 ± 0.2	0.021	0.010	0.70
3 week tail length (cm)	5.2 ± 0.1	5.5 ± 0.1	5.7 ± 0.1	5.7 ± 0.1	0.049	0.037	0.63
6 week mass (g)	15.5 ± 0.5	17.0 ± 0.4	19.1 ± 0.4	18.9 ± 0.3	0.025	0.014	0.67
6 week tail length (cm)	7.0 ± 0.1	7.2 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	0.095	0.029	0.90
10 week mass (g)	19.2 ± 0.6	20.8 ± 0.5	22.6 ± 0.5	22.3 ± 0.4	0.054	0.043	0.56
10 week tail length (cm)	7.8 ± 0.1	8.1 ± 0.1	8.1 ± 0.1	8.1 ± 0.1	0.002	0.001	0.43
12 week mass (g)	20.4 ± 0.6	22.2 ± 0.4	23.7 ± 0.4	23.1 ± 0.3	0.007	0.009	0.25

12 week tail length (cm)	8.0 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.1	0.055	0.025	0.79
Mandible length (mm)	7.9 ± 0.1	8.3 ± 0.1	8.9 ± 0.1	8.8 ± 0.1	0.008	0.002	0.72
Mandible height (mm)	5.6 ± 0.1	6.0 ± 0.1	6.6 ± 0.1	6.5 ± 0.1	0.005	0.002	0.56
Skull (mm)	20.9 ± 0.2	22.2 ± 0.1	22.9 ± 0.1	22.8 ± 0.1	0.001	0.001	0.38
Humerus (mm)	11.1 ± 0.1	11.5 ± 0.1	11.7 ± 0.1	11.7 ± 0.1	0.003	0.001	0.57
Ulna/Radius (mm)	13.0 ± 0.1	13.3 ± 0.1	13.6 ± 0.1	13.6 ± 0.1	0.007	0.002	0.67
Femur (mm)	13.7 ± 0.1	14.2 ± 0.1	14.5 ± 0.1	14.4 ± 0.1	0.002	0.001	0.37
Tibia (mm)	16.0 ± 0.1	16.5 ± 0.1	17.0 ± 0.1	17.0 ± 0.1	0.001	0.001	0.62
Pelvic girdle (mm)	17.7 ± 0.1	18.6 ± 0.1	18.7 ± 0.1	18.7 ± 0.1	0.001	0.001	0.97

¹Values are least square means (\pm standard error), from a model including the effects of *Pappa2* genotype, *Sp7-Cre* genotype, the interaction between *Pappa2* genotype and *Sp7-Cre* genotype, sex and litter identity.

Table 2. Comparison of phenotypic effects of constitutive and osteoblast-specific *Pappa2* deletion.

Trait	Sample sizes				Difference	Difference	P-value for
	<i>Pappa2</i> ^{wt/KO}	<i>Pappa2</i> ^{KO/KO}	<i>Pappa2</i> ^{wt/fl} ; <i>Sp7-Cre</i>	<i>Pappa2</i> ^{fl/fl} ; <i>Sp7-Cre</i>	between <i>Pappa2</i> ^{wt/KO} and <i>Pappa2</i> ^{KO/KO} mice ¹	between <i>Pappa2</i> ^{wt/fl} ; <i>Sp7-Cre</i> and <i>Pappa2</i> ^{fl/fl} ; <i>Sp7-Cre</i> mice ¹	contrast between constitutive and osteoblast- specific effect
3 week mass	53	24	22	15	0.48 ± 0.15	0.56 ± 0.20	0.74
3 week tail length	16	8	22	15	1.14 ± 0.31	0.38 ± 0.24	0.05
6 week mass	48	22	22	15	1.19 ± 0.20	0.62 ± 0.27	0.09
6 week tail length	16	7	22	15	1.82 ± 0.30	0.40 ± 0.22	0.0002
10 week mass	53	22	21	11	1.07 ± 0.14	0.49 ± 0.21	0.03
10 week tail length	16	7	21	11	4.73 ± 0.36	0.86 ± 0.29	0.0001
Mandible length	39	20	21	10	2.19 ± 0.20	0.80 ± 0.27	0.0001
Mandible height	38	20	21	10	2.67 ± 0.21	0.77 ± 0.29	0.0001

Skull	39	20	13	5	1.94 ± 0.24	1.70 ± 0.44	0.63
Humerus	39	20	21	11	1.06 ± 0.21	0.78 ± 0.27	0.40
Ulna/radius	39	20	21	11	0.54 ± 0.19	0.66 ± 0.25	0.70
Femur	39	20	21	11	1.70 ± 0.22	0.85 ± 0.28	0.02
Tibia	39	20	21	11	1.11 ± 0.22	0.94 ± 0.28	0.63
Pelvic girdle	39	20	17	11	1.68 ± 0.23	1.13 ± 0.32	0.17

¹Values are in phenotypic standard deviation units for all traits, and are estimates (\pm standard error) of the differences between the least squares means of genotypes, from a model including the effects of experiment (i.e., osteoblast-specific or constitutive deletion), litter identity and sex.