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

Neilab Amiri, Julian K. Christians *

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

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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.

1. 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.

2. Materials and methods

2.1. 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).

Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PAPP-A2, pregnancy associated plasma protein-A2.

* Corresponding author.
E-mail address: jkchrist@sfu.ca (J.K. Christians).

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2.2. 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 are 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 the 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; and $Pappa2^{fl/fl}$; *Sp7-Cre*: 5 males and 6 females.

2.3. 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.

2.4. 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′-CAGCAAAGGAAATTTGTGCT-3′), KO_exon2 (5′-GGTCAAATGAAACTTCCCTCC-3′), KO_dist2 (5′-CTCTTGCATGCCTCCACTAC-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′-GCGGTCTGGCAGTAAAAA CTATC-3′), Cre_B (5′-GTGAAACAGCATTGCTGTCACTT-3′), Cre_+ve_A (5′-CTAGGCCACAGAATTGAAAGATCT-3′), Cre_+ve_B (5′-GTAGGTGG AAATTCTAGCATCATCC-3′).

2.5. 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.

2.6. 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).

2.7. 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 h. Femorotibial joints were subsequently decalcified in 10% EDTA and embedded in paraffin. Sections were deparaffinized and rehydrated according to standard protocols and heat-antigen retrieval was performed in a 10 mM citrate buffer with 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)