Pregnancy Associated Plasma Protein A2 (PAPP-A2) Affects Bone Size and Shape and Contributes to Natural Variation in Postnatal Growth in Mice

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Abstract

Pregnancy associated plasma protein A2 (PAPP-A2) is a protease of insulin-like growth factor binding protein 5 and is receiving increasing attention for its roles in pregnancy and postnatal growth. The goals of the present study were to characterize the effects of PAPP-A2 deletion on bone size and shape in mice at 10 weeks of age, and to determine whether Pappa2 is the gene responsible for a previously-identified quantitative trait locus (QTL) contributing to natural variation in postnatal growth in mice. Mice homozygous for constitutive PAPP-A2 deletion were lighter than wild-type littermates, and had smaller mandible dimensions and shorter skull, humerus, femur, tibia, pelvic girdle, and tail bone. Furthermore, PAPP-A2 deletion reduced mandible dimensions and the lengths of the skull, femur, pelvic girdle, and tail bone more than would be expected due to the effect on body mass. In addition to its effects on bone size, PAPP-A2 deficiency also altered the shape of the mandible and pelvic girdle, as assessed by geometric morphometrics. Mice homozygous for the PAPP-A2 deletion had less deep mandibles, and pelvic girdles with a more feminine shape. Using a quantitative complementation test, we confirmed that Pappa2 is responsible for the effects of the previously-identified QTL, demonstrating that natural variation in the Pappa2 gene contributes to variation in postnatal growth in mice. If similar functional variation in the Pappa2 gene exists in other species, effects of this variation on the shape of the pelvic girdle might explain the previously-reported associations between Pappa2 SNPs and developmental dysplasia of the hip in humans, and birth in cattle.

Introduction

Insulin-like growth factors (IGFs) play a pivotal role in growth and development [1]. The bioavailability of the IGFs is regulated by six IGF binding proteins (IGFBPs), and the release of IGFs and subsequent IGF signaling is achieved through cleavage of the IGFBPs by proteases [1]. Pregnancy associated plasma protein A (PAPP-A) is an IGFBP protease that was initially identified as a circulating protein of placental origin, but has since been shown to play roles outside of pregnancy in cardiovascular disease, the regulation of bone mineral density, skin healing and aging [2,3,4,5,6]. Screening of sequence databases for homologs of PAPP-A subsequently identified PAPP-A2, an IGFBP-5 protease that shares 45% amino acid sequence identity with PAPP-A [7]. While less work has examined PAPP-A2, this protein is receiving increasing attention for its roles in human pregnancy [2,8], reproductive traits in cattle [9,10,11], and postnatal growth in mice [12,13].

The first indication of a role in postnatal growth was our previous work that identified Pappa2 as a candidate for the gene responsible for the effects of a quantitative trait locus (QTL) affecting adult body size in mice. We refined the location of the QTL to a 1 megabase (MB) region that contains only 4 genes, and found that the QTL affected circulating levels of IGFBP-5, the target of PAPP-A2 [12]. Recently, Conover et al. developed PAPP-A2 knock-out mice which are viable and smaller than wild-type [13]. Because IGFBP-5 is known to play a role in bone metabolism [14,15], PAPP-A2 is expected to have a particularly pronounced effect on skeletal growth. Deletion of PAPP-A2 leads to shorter femur length, but no effects on bone mineral density were detected [13]. The QTL described above also affects the length of certain bones [16]. In cattle, associations have been found between SNPs in Pappa2 and difficulty giving birth, potentially through effects on the size or shape of the mother’s pelvis [10]. Others have reported association between developmental dysplasia of the hip and a Pappa2 SNP in humans [17], again suggesting an effect of PAPP-A2 on bone shape.

The goals of the present study were to further characterize the effects of PAPP-A2 deletion on skeletal characters in mice, including not only bone lengths but also bone shapes, and to determine whether Pappa2 is the gene responsible for the QTL described above [12]. Although Pappa2 was previously identified as a candidate for a QTL affecting body size [12], and deletion of PAPP-A2 reduces body size [13], it does not necessarily follow that Pappa2 is responsible for the effects of the QTL; there might be no functional variation in the Pappa2 gene between the two wild-type mouse strains studied in the QTL work. To test whether Pappa2 is the causal gene underlying the QTL, we performed a quantitative
complementation test [18,19,20]. Establishing whether Pappa2 is responsible for the effects of the QTL will shed light on the functional significance of naturally-occurring variation in this gene, and will help to identify functionally important coding and regulatory regions.

Methods

Ethics Statement

All work was carried out in accordance with the guidelines of the Canadian Council on Animal Care and approved by the SFU University Animal Care Committee (protocol 945B-09).

PAPP-A2 Deletion Mice

Conditional PAPP-A2 deletion mice were generated by Oogene Pty. Ltd. (Bentley, Australia), i.e., independently of the previous PAPP-A2 deletion study [13]. The targeting vector was constructed using Oogene’s Pelle plasmid and fragments generated by PCR from C57BL/6 genomic DNA, such that mouse exon 2 (homologous to human exon 3) and a PGK-Neo selection cassette were flanked by LoxP sites (i.e., “floxed”, Fig. 1). Following Cre-mediated deletion of exon 2, splicing of exon 1 to exon 3 is expected to produce an early stop codon. The targeting vector was electroporated into C57BL/6 ES cells and correctly targeted clones were injected into blastocysts for the generation of chimeras. High percentage chimeras were mated with C57BL/6 mice to produce offspring homozygous for the floxed allele.

Deletion of exon 2 and removal of the Neo cassette were achieved by crossing conditional deletion mice to mice with a C57BL/6 background expressing Cre recombinase (hereafter referred to as Cre) under the control of a human cytomegalovirus minimal promoter (Jackson Laboratory stock number 006054). Removal of the floxed region in the progeny was determined by PCR genotyping (described below). In subsequent experiments (described below), we tested whether Cre itself had an effect on phenotype in preliminary analyses in which we included the presence of Cre (yes/no) as a term in the model. The presence of Cre did not have a significant (P < 0.05) effect on body mass or tail length at any age, or on any bone phenotype, in analyses of the effects of PAPP-A2 deletion or the quantitative complementation test, with one exception: the effect of Cre was significant for tail length at 3 weeks of age in the quantitative complementation test, but not in the analyses of the effects of PAPP-A2 deletion. Since the presence of Cre affected only one trait in only one of two experiments, this variable was removed from the model for further analyses.

Mice heterozygous for the constitutive Pappa2 disruption were paired to produce litters in which all three genotypes were present (i.e., homozygous disruption, heterozygous, and homozygous wild-type). For measuring placental and embryonic weights and PAPP-A2 expression, females were checked for seminal plugs the morning following pairing, and plugged females were euthanized with CO2 twelve days later (embryonic day 12.5). We chose to collect females at embryonic day 12.5 to ensure that individual placentae and embryos would be sufficiently large for protein extraction and because PAPP-A2 expression is high at this stage [21,22]. Placentae and embryos were dissected while immersed in 10× PBS, weighed, and frozen at −80°C for quantification of protein levels by Western blotting. The amniotic sac and/or a piece of embryonic tail was collected for PCR genotyping.

To determine birth weight, females were not checked for plugs, but were instead left with males, checked daily three to five hours after the lights were turned on, and new pups were weighed. We anaesthetized the newborn offspring using isofluorane, and collected 2 mm of tail tissue for genotyping. These pups were also earclipped for genotyping at 3 weeks of age and weighed at 3, 6 and 10 weeks of age. Additional mice were not weighed at birth, but earclipped for genotyping at 3 weeks of age, and mass and tail length were measured at 3, 6 and 10 weeks of age.

Bone Measurements

Following sacrifice at 10 weeks of age, mice were frozen and thawed at a later date at which time the skin and internal organs were removed and the carcasses were dried and then exposed to dermestid beetles to remove soft tissue. The following bones were measured: 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, pelvic girdle, and a tail bone (the tenth caudal bone counting from the first bone of the sacrum). Where applicable we measured bones from both sides of each animal and calculated the mean. All

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**Figure 1. Schematic diagram of the targeting vector used in the generation of PAPP-A2 conditional deletion mice.** Shown are the locations of the targeted exon, homology arms, loxP and FRT sites, ampicillin resistance gene (AmpR), phosphoglycerate kinase promoter (PGK), neomycin resistance gene (Neo), bovine growth hormone poly A termination signal (pA), and genotyping primers. The sizes of the primers have been exaggerated for clarity.

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skeletal dimensions were measured with digital calipers (∆0.01 mm) and measurements were performed in triplicate.

**Quantitative Complementation Test**

In addition to linear size measurements, the shapes of the mandible and pelvic girdle were analyzed. Photographs were taken of the left and right mandible, and left and right pelvic girdles. Landmarks were placed on photos using the tpsDig2 program (tps software package, version 2.16; F. James Rohlf; http://life.bio.sunysb.edu/morph/), with 12 landmarks for mandibles and 14 for the pelvic girdles. The tpsDig2 software was also used to create outline images representing the curvature of the bones, used to visualize differences in bone shape. The tpsUtil program (tps utility software, version 1.49; F. James Rohlf; http://life.bio.sunysb.edu/morph/) was used to form the tps input files.

Procrustes analysis was performed on landmark data using MorphoJ [version 1.05a; C.P. Klingenberg; http://www.flywings.org.uk/MorphoJ_page.htm [23]]. The Procrustes analysis uses superimposition to reorient and scale the imported landmark coordinates so they are aligned in the same position and at approximately the same size in order to quantify variation at each landmark coordinate regardless of size or original positioning in the photograph [24]. This analysis also accounts for symmetry between the left and right bones, and left and right bones from a given mouse were subsequently averaged. The Procrustes analysis yields centroid size, a measure of size, and Procrustes coordinates, which provide information on shape, independent of size. Procrustes coordinates were analysed using Principal Component Analysis (PCA) to reduce the dimensionality of the data, i.e., to reduce the multiple distances between coordinates to a smaller number of variables. PCA was performed using the pooled within-group covariance (within litter, sex and genotype). PCA scores, as well as centroid data, were exported from MorphoJ into SAS, Version 9.3 (SAS Institute Inc., Cary, NC) for further analyses. To facilitate comparisons between PAPP-A2 deletion mice and mice from the quantitative complementation test (described below), mice from both experiments were combined for the PCA such that principal component scores describe the same variation in shape for all mice.

**Quantitative Complementation Test**

A quantitative complementation test is used to test whether a gene is the causal agent underlying a QTL. The logic is as follows: An individual heterozygous for a knock-out of gene X is crossed with an individual heterozygous for two QTL alleles, producing offspring that vary both for the knock-out and for the QTL alleles. These offspring are measured for the phenotype of interest, i.e., the trait known to be affected by the QTL. If gene X is not the gene responsible for the QTL, and if gene X does not interact with the gene responsible for the QTL, there will be an effect of QTL allele on the phenotype, and there may be an effect of the knock-out, i.e., the phenotypic effect of the QTL allele will depend on whether gene X is knocked out or not (see Table 1).

In previous work, we introgressed the C57BL/6 allele of a chromosome 1 QTL into the DBA/2 background to produce a line segregating for an approximately 1 MB region containing the QTL, including Pappa2 [12]. This line was at backcross generation 14 at the time of this study. The quantitative complementation test was carried out by crossing mice heterozygous for the QTL allele with mice heterozygous for constitutive Pappa2 disruption (described above). The offspring inherited either the C57BL/6 allele or DBA/2 allele of the introgressed segment from their QTL line parent, and either the Pappa2 disruption allele or the wild-type allele from their disruption parent. Outside of the 1 MB QTL region, the offspring all had the same C57BL/6 or DBA/2 heterozygous genetic background. Offspring were ear-clipped for genotyping at 3 weeks of age, and mass and tail length were measured at 3, 6 and 10 weeks of age.

**Genotyping**

Genotyping was performed by standard methods. PCR genotyping was used for the determination of (a) Pappa2 alleles (wild-type vs. disrupted), (b) QTL alleles (DBA/2 vs. C57BL/6) and (c) the presence/absence of the Cre recombinase gene. Genotyping for the Pappa2 allele used 4 primers at locations shown in Fig. 1. These primers were designed to yield bands of different sizes for the four possible alleles (408 bp for wild-type, 308 bp for deleted, 341 bp for floxed with the Neo selection cassette present, and 547 bp for floxed with the Neo selection cassette absent; only the first two alleles were present in this study). Genotyping of QTL alleles was performed by amplifying two fragments at either end of the QTL region. These fragments were then digested by XbaI which cut only one of the alleles due to a SNP in the restriction site, generating a restriction fragment length polymorphism. The genotyping reactions for Cre included two primer pairs recommended by the Jackson Laboratory: one to amplify a fragment from the Cre transgene and one 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 provided in Table 2.

**Protein Extraction and Western Blotting**

Placentae and embryos were homogenized in 2 mL of T-PER™ Tissue Protein Extraction Reagent (PIERCE, Rockford, IL), incubated on ice for 7 minutes and then centrifuged at 10000 g for 5 minutes at 4°C. Complete protease inhibitor cocktail (Roche Applied Sciences) was added to the supernatant, which was then stored at −20°C.

Placental and embryonic samples containing 40 μg of protein were mixed with SDS loading buffer, boiled and run through a 4% stacking and 8% separating polyacrylamide gel for 60 minutes. The gel was equilibrated in transfer buffer and transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked for one hour at room temperature in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, Nebraska), incubated for one hour at room temperature (placentae) or overnight at 4°C (embryos) in a solution containing 1:500 monoclonal mouse anti-actin (CLT9001; Cedarlane, Burlington ON) and 1:5000 polyclonal goat-anti-human PAPP-A2 antibody (AF1668; R&D Systems, Minneapolis, MN) diluted in Odyssey Blocking Buffer (Li-Cor Biosciences, Lincoln, NE) and 0.1% Tween-20 (Sigma, ON, Canada), washed 4 times, and incubated in a solution containing 1:10000 fluorescently-labelled IRDye 800 anti-goat and IRDye 680 anti-mouse secondary antibodies (Li-Cor Biosciences, Lincoln, NE) diluted in Odyssey Blocking Buffer, 0.1% Tween-20 and 0.1% SDS for 45 minutes with gentle shaking. The membranes were again washed 4 times. Membranes were visualized using the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

The secondary dyes fluoresce at different wavelengths (anti-goat at 800 nm and anti-mouse at 700 nm), so we were able to simultaneously quantify the intensity...
PAPP-A2 Deletion

Results

PAPP-A2 Deletion

Following Cre-mediated excision of the floxed region, bands of the expected size were obtained from PCR (data not shown) and PAPP-A2 protein was not detectable in placentae or embryos homozygous for the disrupted allele, and PAPP-A2 levels in heterozygotes were lower than those in homozygous wild-types (Fig. 2). In crosses between mice heterozygous for the disruption, the offspring genotypes did not differ from the expected Mendelian ratios in utero, at birth, or at weaning (Table 3), suggesting no effect of PAPP-A2 deletion on viability.

Effects of PAPP-A2 Deletion on Mass and Size

At embryonic day 12.5, there was no detectable effect of PAPP-A2 deletion on placental or embryonic mass (Table 4). There was marginally non-significant variation among genotypes in birth mass ($F_{2,23} = 2.54; P = 0.09$), with a trend towards lower birth mass in pups homozygous for the PAPP-A2 deletion (Table 4). In MANOVA of size at 3, 6, and 10 weeks of age, the effect of PAPP-A2 genotype was significant for both body mass (Wilks' Lambda $F_{6,38} = 8.75; P = 0.0001$), and so further single variable tests were performed. The effect of PAPP-A2 deletion generally did not depend on sex; the only significant genotype by sex interaction term from the model, variation among genotypes in birth mass (Wilks' Lambda $F_{6,38} = 8.75; P = 0.0001$) and tail length (Wilks' Lambda $F_{6,38} = 8.75; P = 0.0001$), and so further single variable tests were performed. The effect of PAPP-A2 deletion generally did not depend on sex; the only significant genotype by sex interaction was not significant for any trait and so was removed from the model. Consistent with the highly significant effect on 10 week body mass and tail length, PAPP-A2 deletion had a significant effect on mandible dimensions and

Table 1. Predicted phenotypic comparisons in offspring from the quantitative complementation test if Pappa2 is or is not the quantitative trait gene (QTG), i.e., the gene responsible for the effect of the QTL.

| Offspring of four possible genotypes: the genotype at the QTG will be: | C57BL6/disrupted | DBA2/disrupted | C57BL6/DBA2 | DBA2/C57BL6 |
|---|---|---|---|---|
| If Pappa2 is responsible for the effect of the QTL, the genotype at the QTG will be: | C57BL6/C57BL6 | DBA2/C57BL6 | C57BL6/C57BL6 | DBA2/C57BL6 |

Prediction: The phenotypic difference between C57BL6/disrupted and DBA2/disrupted will be greater than the phenotypic difference between C57BL6/C57BL6 and DBA2/C57BL6. Little difference is expected between the C57BL6/C57BL6 and DBA2/C57BL6 genotypes because the C57BL6 allele is partially dominant [12].

If Pappa2 is not responsible for the effect of the QTL, the genotype at the QTG will be: C57BL6/C57BL6.

Prediction: The phenotypic difference between C57BL6/disrupted-Pappa2 and DBA2/disrupted-Pappa2 will be equal to the phenotypic difference between C57BL6/intact-Pappa2 and DBA2/intact-Pappa2.

Table 2. Primer sequences.

| Pappa2 allele genotyping | KO proximal | CAGCAGAGGAAATGGTGTCT |
|---|---|---|
| KO distal | ATCCCTAGCTCTCTCTGTAT |
| KO exon | CTTCGATGATGGAGACTGCT |
| KO neo | GCATTGGGAAGACAAATAGCA |
| QTL allele genotyping | Proximal forward | GTGTGTTGGTGTGAGACATT |
| Proximal reverse | AACATAAGGTGACATGCTTCCA |
| Distal forward | CCTGTCGTCAAGCTGAAAA |
| Distal reverse | GTTCATGGTCTCTTCATACC |
| Cre genotyping | Cre forward | GCGGTCCTGGCGAGAAACTATC |
| Cre reverse | GTGAACAGCTTTGGTGTCC |
| Positive control forward | CTAGGCCACAGAATTGAAAGATCT |
| Positive control reverse | TTAGTGGAAATTCTAGCATC |

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References

[12]
the lengths of the skull, humerus, femur, tibia, pelvic girdle, and tail bone (Table 4); the effect on the length of the ulna/radius was marginally non-significant ($F_{2,50} = 2.71; P = 0.08$). In all cases, mice homozygous for the PAPP-A2 deletion had shorter bones, while bone size was similar in heterozygotes and homozygous wild-types. To determine whether effects on bone dimensions were due to a general effect on size, or whether certain bones were affected disproportionately, we repeated the analysis while controlling for 10 week body mass (by including body mass as a covariate). As with measures of size, which is a measure of overall bone size. Consistent with the linear measures described above, deletion of PAPP-A2 had a highly significant effect on the centroid size of both the mandible and pelvic girdle ($P < 0.0001$ in both cases), with mice homozygous for Papp2 disruption having smaller bones, and heterozygotes and homozygous wild-type mice having bones of similar size.

In contrast to the effects of PAPP-A2 deletion on bone lengths, there was no effect of Papp2 genotype on the dry masses of the stomach, spleen, pancreas, kidneys, liver, lungs or heart (MANOVA Wilks’ Lambda $F_{16,100} = 1.42; P = 0.15$). There was also significant variation among genotypes in mandible PC2 ($F_{2,50} = 8.70; P = 0.0006$) but not PC1 ($F_{2,50} = 0.87; P = 0.43$). As with measures of size, shape in heterozygous mice was much more similar to homozygous wild-types than to mice homozygous for Papp2 disruption. Pelvic girdle PC1 largely reflects variation in the shape of the ischium, as well as the length of the pubis and, to a lesser extent, the width of the end of the ilium (Fig. 3B). Mice homozygous for the Papp2 disruption have more negative values of PC2 (Fig. 3A), which indicates a smaller distance between the tips of the angular and coronoid processes. Pelvic girdle PC2 includes variation in the distance between the tips of the angular and coronoid processes (Fig. 3A). Including sex and litter as terms in the model, there was significant variation among Papp2 genotypes in mandible PC2 ($F_{2,50} = 8.70; P = 0.0006$) but not PC1 ($F_{2,50} = 0.87; P = 0.43$). As with measures of size, shape in heterozygous mice was much more similar to homozygous wild-types than to mice homozygous for Papp2 disruption. Pelvic girdle PC1 largely reflects variation in the shape of the ischium, as well as the length of the pubis and, to a lesser extent, the width of the end of the ilium (Fig. 3B). Mice homozygous for the Papp2 disruption have more negative values of PC2 (Fig. 3A), which indicates a smaller distance between the tips of the angular and coronoid processes, i.e., a less deep bone.

There was also significant variation among Papp2 genotypes in pelvic girdle PC1 ($F_{2,50} = 9.26; P = 0.0003$), but not pelvic girdle PC2 ($F_{2,50} = 0.13; P = 0.88$), including sex and litter as terms in the model. As with above, shape in heterozygous mice was much more similar to homozygous wild-types than to mice homozygous for Papp2 disruption. Pelvic girdle PC1 largely reflects variation in the shape of the ischium, as well as the length of the pubis and, to a lesser extent, the width of the end of the ilium (Fig. 3B). This captured the sexual dimorphism in pelvic girdle shape, with little overlap in PC1 between males and females. Mice homozygous for the Papp2 disruption had significantly lower PC1 scores, suggesting a more “female” shape, i.e., male knock-outs were

| Stage | $-/-$ | $-/+ | +/+ | P-value* |
|-------|------|------|------|----------|
| E125 | 3    | 5    | 6    | 0.30     |
| Birth | 14   | 37   | 11   | 0.27     |
| Weaning | 24 | 53   | 18   | 0.36     |

*Chi-square test of null hypothesis that genotypes are in 1:2:1 ratio.

The sample measured at weaning and into adulthood includes 50 females and 45 males.

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Effects of PAPP-A2 Deletion on Bone Shape

The Procrustes analysis used to remove variation in position, orientation and size from bone landmark data yielded centroid size, which is a measure of overall bone size. Consistent with the linear measures described above, deletion of PAPP-A2 had a highly significant effect on the centroid size of both the mandible and pelvic girdle ($P < 0.0001$ in both cases), with mice homozygous for Papp2 disruption having smaller bones, and heterozygotes and homozygous wild-type mice having bones of similar size.

In Principal Component Analysis of the Procrustes coordinates, the first two principal components explained 47% and 34% of the variation in size-independent shape of mandibles and pelvic girdles, respectively; subsequent principal components each explained less than 12% of the variation. Plotting the second principal component (PC2) against the first (PC1) illustrates differences in bone shape between mice homozygous for Papp2 disruption and those with at least one functional copy of Papp2, as well as differences between sexes (Figs. 3A and 3B). Including sex and litter as terms in the model, there was significant variation among Papp2 genotypes in mandible PC2 ($F_{2,50} = 8.70; P = 0.0006$) but not PC1 ($F_{2,50} = 0.87; P = 0.43$). As with measures of size, shape in heterozygous mice was much more similar to homozygous wild-types than to mice homozygous for Papp2 disruption. In mandibles, PC2 includes variation in the distance between the tips of the angular and coronoid processes (Fig. 3A). Mice homozygous for the Papp2 disruption have more negative values of PC2 (Fig. 3A), which indicates a smaller distance between the tips of the angular and coronoid processes, i.e., a less deep bone.

There was also significant variation among Papp2 genotypes in pelvic girdle PC1 ($F_{2,50} = 9.26; P = 0.0003$), but not pelvic girdle PC2 ($F_{2,50} = 0.13; P = 0.88$), including sex and litter as terms in the model. As with above, shape in heterozygous mice was much more similar to homozygous wild-types than to mice homozygous for Papp2 disruption. Pelvic girdle PC1 largely reflects variation in the shape of the ischium, as well as the length of the pubis and, to a lesser extent, the width of the end of the ilium (Fig. 3B). This captured the sexual dimorphism in pelvic girdle shape, with little overlap in PC1 between males and females. Mice homozygous for the Papp2 disruption had significantly lower PC1 scores, suggesting a more “female” shape, i.e., male knock-outs were
A quantitative complementation test was performed to test whether Pappa2 is the gene responsible for a previously-described QTL affecting body size. The prediction is that, if Pappa2 is the gene responsible for the effect of the QTL, the phenotypic difference between QTL genotypes will be greater in mice who inherited a disrupted Pappa2 allele than in mice who inherited a wild-type Pappa2 allele from their heterozygous disruption parent (Table 1). The mean values fit this prediction for tail length and body mass at 3, 6, and 10 weeks of age (Fig. 4), with mice inheriting a DBA/2 allele and a disrupted Pappa2 allele having the lowest values. In MANOVA, the statistical interaction between Pappa2 disruption genotype and QTL genotype was significant for body mass (Wilks’ Lambda $F_{3,200} = 3.60; P = 0.014$) and tail length (Wilks’ Lambda $F_{3,196} = 6.00; P = 0.0006$) at 3, 6 and 10 weeks. However, in single variable tests, the interaction was significant only for tail length at 3, 6 and 10 weeks ($F_{1,201} = 6.60; P = 0.01$, $F_{1,201} = 7.93; P = 0.005$, and $F_{1,200} = 15.43; P = 0.0001$, respectively) and for body mass at 3 weeks ($F_{1,201} = 9.71; P = 0.002$), but not at 6 or 10 weeks ($F_{1,201} = 1.81; P = 0.18$ and $F_{1,201} = 0.11; P = 0.74$, respectively). Litter and sex were also included as terms in the model in these analyses. The quantitative complementation test therefore established that Pappa2 is responsible for at least part of the effect of the QTL.

The interaction between Pappa2 disruption genotype and QTL genotype was marginally non-significant for the bones of the skull (skull and mandible dimensions; MANOVA Wilks’ Lambda $F_{3,194} = 2.22; P = 0.09$) and not significant for the long bones (humerus, radius/ulna, femur and tibia; MANOVA Wilks’ Lambda $F_{4,194} = 0.20; P = 0.94$). In single variable analyses, the quantitative complementation test was significant for mandible length ($F_{1,196} = 5.66; P = 0.02$), the length of the pelvic girdle ($F_{1,196} = 3.90; P = 0.05$) and was marginally non-significant for mandible height ($F_{1,196} = 3.60; P = 0.06$), and the centroid sizes of the mandible ($F_{1,196} = 3.55; P = 0.06$) and pelvic girdle ($F_{1,196} = 2.83; P = 0.09$), but not significant for any other linear bone measurements or shape measurements (data not shown). While the interaction was not significant, the expected trend (see Table 1) was observed for all linear bone measurements.

### Discussion

We have shown that deletion of PAPP-A2 reduces the lengths of some bones beyond what is expected due to reduced body size, and also affects bone shape. In particular, we found changes in pelvic girdle shape, providing a potential explanation for the previously-reported associations between Pappa2 SNPs and developmental dysplasia of the hip in humans [17] and birthing in cattle [10]. We found a substantial sexual dimorphism in pelvic girdle shape, consistent with previous work [25], and the pelvic girdle of mice homozygous for the Pappa2 disruption had a more feminine shape, in addition to being disproportionately small for a given body size. Natural variation in the Pappa2 gene could therefore increase the risk of birthing complications through a reduction in PAPP-A2 expression or activity leading to reduced pelvic girdle size and/or to an extreme female shape that was suboptimal for giving birth. Alternatively, a SNP that increased PAPP-A2 activity or expression might lead to a pelvic girdle with a more masculine shape, which could also cause difficulties during birth.

Deletion of PAPP-A2 also affected the shape of the mandible, with mice homozygous for the disruption having a smaller distance between the tips of the angular and coronoid processes, i.e., a less deep bone. We therefore hypothesize that natural variation in Pappa2 is a potential source of variation for evolutionary adaptations in mandible shape, e.g., in response to digging behaviour [26] or diet [27,28]. For example, shallower mandibles have been associated with a more omnivorous diet, whereas deeper, more robust mandibles have been associated with diets which required more powerful mastication [27].
Figure 3. Effects of gene disruption on mandible and pelvic girdle shape. In the scatterplots of the first and second principal components of (A) mandible landmark data and (B) pelvic girdle landmark data, black/red symbols are females, open/blue symbols are males, squares are homozygous wild-type, diamonds are heterozygotes and circles are homozygous Pappa2 disruption. To highlight the effects of disruption, symbols for homozygote knock-out females are red and those for homozygote knock-out males are blue. Outlines above the graph illustrate variation in shape measured by the first principal component, and represent extremes in shape (PC1 = −0.1 and 0.1) to facilitate visualization. Outlines to the right of the graph illustrate variation in the second principal component. Dots on the outlines indicate the location of landmarks. Note that analyses are carried out on the locations of landmarks, and outlines are drawn only to visualize changes in shape; outlines should therefore be interpreted with caution. Because of broken, damaged or missing bones, sample sizes were reduced compared to analyses of body mass, and a total of 61 mandibles and 71 pelvic girdles were analysed.

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Effects of PAPP-A2 on Bone Size and Shape

- Mass at 3 weeks (g)
- Tail length at 3 weeks (cm)

- Mass at 6 weeks (g)
- Tail length at 6 weeks (cm)

- Mass at 10 weeks (g)
- Tail length at 10 weeks (cm)

*Pappa2 disruption genotype*
Effects of PAPP-A2 on Bone Size and Shape

Unlikely the study by Conover et al. [13], we did not find that the effects of PAPP-A2 deletion differed between the sexes. Despite high PAPP-A2 expression in the placenta [22], we did not find prenatal effects of PAPP-A2 deletion, although there was a non-significant trend towards lower birth mass in pups homozygous for the Poppa2 disruption. A lack of effect on birth mass is consistent with the previous study of PAPP-A2 knock-outs [13], as well as our previous work examining variation in placental PAPP-A2 expression between QTL alleles [21].

The specific mechanisms through which the deletion of PAPP-A2 affects bone size and shape requires further study, but we expect that deletion of PAPP-A2 will lead to a reduction in IGFBP-5 proteolytic activity and consequently higher levels of intact IGFBP-5 and lower free IGF levels, leading to reduced growth. In addition to the effects of IGFBP-5 on IGF availability, IGFBP-5 may also have IGF-independent effects [15]. Because the IGFBPs are thought to have important local actions in bone [14,15], the consequences of PAPP-A2 deletion may be due to local effects, and so PAPP-A2 deletion would not necessarily be expected to influence circulating levels of IGFBP-5 or IGFs.

In addition to characterizing the effects of PAPP-A2 deletion on skeletal phenotypes, the quantitative complementation test established that Poppa2 is responsible for at least some of the effect of a previously-described QTL [12]. The quantitative complementation test showed significant interactions for tail length at 3, 6 and 10 weeks and body mass at 3 weeks, and was significant or marginally non-significant for measures of mandible and pelvic girdle size, but was not significant for body mass at 6 or 10 weeks or the sizes of any other bones. These results are consistent with previous observations of the effects of the QTL. Previously, we have found that the effects of the QTL on body mass are smaller than those on tail length [29-30]. Furthermore, we have only found effects of the QTL on the dimensions of the mandible and tail bone, but not other long bones, although this earlier study used a different measure of the pelvic girdle [16]. It is not clear whether the lack of significance in the quantitative complementation tests for bones other than mandible and pelvic girdle represents a real lack of effect of the QTL or low statistical power. The quantitative complementation test therefore demonstrates that natural variation in the Poppa2 gene, which exists between the inbred lines of mice used in our previous QTL mapping work, contributes to variation in at least some aspects of body size in mice. This is one of the first instances in which the gene responsible for a QTL affecting growth has been identified. This QTL is due to variation between the C57BL/6 and DBA/2 alleles, and both of these strains have been sequenced extensively. Many if not all of the candidate variants between the alleles are therefore already known, and can be used to investigate functionally important codon and regulatory regions. Although it is not clear whether the QTL is due to regulatory or coding sequence variation, we have previously found differences in PAPP-A2 expression levels between QTL genotypes [21].

In conclusion, our results show that natural variation in the Poppa2 gene can contribute to variation in postnatal growth. If similar functional variation in the Poppa2 gene exists in other species, effects of this variation on the shape of the pelvic girdle could potentially explain the previously-reported associations between SNPs in Poppa2 and pelvic bone-related phenotypes such as hip dysplasia and birthling ease.

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Author Contributions
Conceived and designed the experiments: JKC. Performed the experiments: JKC SHYF. Analyzed the data: JKC DRDZ. Wrote the paper: JKC DRDZ SHYF.

References
1. Bunn RC, Foulkes JL (2003) Insulin-like growth factor binding protein proteolysis. Trends Endocrinol Met 14: 176–181.
2. Christians JK, Grudin A (2010) Altered levels of insulin-like growth factor binding protein proteases in preclampsia and intrauterine growth restriction. Prenat Diag 30: 813–820.
3. Chen BK, Leiferman KM, Pittrow KB, Overgaard MT, Oveig C, et al. (2003) Localization and regulation of pregnancy-associated plasma protein A expression in healing human skin. J Clin Endocrinol Metab 88: 4465–4471.
4. Conover CA, Bale LK (2007) Loss of pregnancy-associated plasma protein A extends lifespan in mice. Aging Cell 6: 727–729.
5. Kashi JC, Holt DW (2006) Pregnancy-associated plasma protein-A and cardiovascular risk. Eur Heart J 27: 1637–1639.
6. Tanner SJ, Hefferan TE, Rosen CJ, Conover CA (2008) Impact of pregnancy-associated plasma protein-A deletion on the adult murine skeleton. J Bone Miner Res 23: 655–662.
7. Overgaard MT, Bolth HB, Laursen LS, Sottrup-Jensen L, Conover CA, et al. (2001) Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 protease. J Biol Chem 276: 21849–21853.
8. Yan XL, Baxter RC, Firth SM (2010) Involvement of pregnancy-associated plasma protein-A2 in insulin-like growth factor (IGF) binding protein-5 proteolysis during pregnancy: A potential mechanism for increasing IGF bioavailability. J Clin Endocrinol Metab 95: 1412–1420.
9. Luna-Navarre P, Rincon G, Medrano JF, Riley DG, Chase CC Jr, et al. (2011) Single nucleotide polymorphisms in the growth hormone-insulin-like growth factor axis in straightbred and crossbred Angus, Brahman, and Romosinuano heifers: Population genetic analyses and association of genotypes with reproductive phenotypes. J Anim Sci 89: 926–938.
10. Weikrauningle S, Rincon G, Medrano JF (2011) Variants in the pregnancy-associated plasma protein-A2 gene on Bos taurus autosome 16 are associated with daughter calving ease and productive life in Holstein cattle. J Dairy Sci 94: 1552–1558.
11. Luna-Navarre P, Rincon G, Medrano JF, Riley DG, Chase CC Jr, et al. (2012) Identification of one polymorphism from the PAPP-A2 gene associated to fertility in Romosinuano beef heifers raised under a subtropical environment. Revista Mexicana De Ciencias Pecuarias 3: 185–200.
12. Christians JK, Hoedlch A, Gesuale PD (2006) 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: 1547–1553.
13. Conover CA, Bolth HB, Bale LK, Clifton KB, Grell JA, et al. (2011) Pregnancy-associated plasma protein-A2 (PAPP-A2): Tissue expression and biological consequences of gene knockout in mice. Endocrinology 152: 2837–2844.
14. Conover CA (2008) Insulin-like growth factor-binding proteins and bone metabolism. Am J Physiol - Endoc M 294: E10–E14.
15. Govoni KE, Baylink DJ, Mohan S (2005) The multi-functional role of IGF-I in growth factor binding proteins in bone. Pediatr Nephrol 20: 261–268.
16. Christians JK, Senger LC (2007) Fine mapping discover pleiotropic growth quantitative trait locus into linked loci. Mamm Genome 18: 240–245.
17. Jia J, Li L, Zhao Q, Zhang L, Ru J, et al. (2012) Association of a single nucleotide polymorphism in pregnancy-associated plasma protein-A2 with...
developmental dysplasia of the hip: a case-control study. Osteoarth Cartilage 20: 60–63.
18. Cheverud JM, Fawcett GL, Jarvis JP, Norgard EA, Pavlicev M, et al. (2010) Calpain-10 is a component of the obesity-related quantitative trait locus Adip1. J Lipid Res 51: 907–913.
19. Christians JK, Keightley PD (2005) Behavioural genetics: Finding genes that cause complex trait variation. Curr Biol 15: R19–R21.
20. Yalcin B, Willis-Owen SAG, Fullerton J, Meeasq A, Deacon RM, et al. (2004) Genetic dissection of a behavioral quantitative trait locus shows that Rgs2 modulates anxiety in mice. Nat Genet 36: 1197–1202.
21. Wagner PK, Christians JK (2010) Altered placental expression of PAPP-A2 does not affect birth weight in mice. Reprod Biol Endocrin 8: 90.
22. Wang J, Qiu Q, Haidar M, Bell M, Gruslin A, et al. (2009) Expression of pregnancy-associated plasma protein A2 during pregnancy in human and mouse. J Endocrinol 202: 337–345.
23. Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics. Mol Ecol Resour 11: 353–357.
24. Klingenberg CP (2010) Evolution and development of shape: integrating quantitative approaches. Nat Rev Genet 11: 623–635.
25. Schutz H, Donovan ER, Hayes JP (2009) Effects of parity on pelvic size and shape dimorphism in M. J Morphol 270: 834–842.
26. Alvarez A, Ivan Perez S, Verzi DH (2011) Ecological and phylogentic influence on mandible shape variation of South American caviomorph rodents (Rodentia: Hystricormorpha). Biol J Linn Soc 102: 828–837.
27. Renaud S, Michaux JK (2003) Adaptive latitudinal trends in the mandible shape of Apodemus wood mice. J Biogeogr 30: 1617–1628.
28. Monteiro LR, Duarte LC, dos Reis SF (2003) Environmental correlates of geographical variation in skull and mandible shape of the punare rat Thrichomys apereoides (Rodentia: Echimyidae). J Zool 261: 47–57.
29. Christians JK, Bingham V, Oliver F, Heath TT, Keightley PD (2003) Characterization of a QTL affecting skeletal size in mice. Mammalian Genome 14: 173–183.
30. Christians JK, Keightley PD (2004) Fine mapping of a murine growth locus to a 1.4-cM region and resolution of linked QTL. Mammalian Genome 15: 482–491.