Disruption of insulin-like growth factor-II imprinting during embryonic development rescues the dwarf phenotype of mice null for pregnancy-associated plasma protein-A

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Abstract

Pregnancy-associated plasma protein-A (PAPP-A), an insulin-like growth factor-binding protein (IGFBP) protease, increases insulin-like growth factor (IGF) activity through cleavage of inhibitory IGFBP-4 and the consequent release of IGF peptide for receptor activation. Mice homozygous for targeted disruption of the PAPP-A gene are born as proportional dwarfs and exhibit retarded bone ossification during fetal development. Phenotype and *in vitro* data support a model in which decreased IGF-II bioavailability during embryogenesis results in growth retardation and reduction in overall body size. To test the hypothesis that an increase in IGF-II during embryogenesis would overcome the growth deficiencies, PAPP-A-null mice were crossed with $\Delta H19$ mutant mice, which have increased IGF-II expression and fetal overgrowth due

to disruption of *IgfII* imprinting. $\Delta H19$ mutant mice were 126% and PAPP-A-null mice were 74% the size of controls at birth. These size differences were evident at embryonic day 16.5. Importantly, double mutants were indistinguishable from controls both in terms of size and skeletal development. Body size programmed during embryo development persisted post-natally. Thus, disruption of *IgfII* imprinting and consequent elevation in IGF-II during fetal development was associated with rescue of the dwarf phenotype and ossification defects of PAPP-A-null mice. These data provide strong genetic evidence that PAPP-A plays an essential role in determining IGF-II bioavailability for optimal fetal growth and development.

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Introduction

Insulin-like growth factor (IGF)-II is a major determinant of fetal growth. Targeted disruption of the mouse *IgfII* gene produces proportional dwarfs, which are 60% of normal birthweight (DeChiara *et al.* 1990). The onset of the mutational effect leading to this growth deficiency occurs during early embryogenesis due to loss of the growth-promoting function of IGF-II mediated through signaling via type I IGF and insulin receptors (Baker *et al.* 1993, Louvi *et al.* 1997, Burns & Hassan 2001).

IGF action is determined not only by ligand and receptors but also by a family of IGF binding proteins (IGFBP-1 through -6) that, in turn, are regulated by specific proteases (Shimasaki & Ling 1991, Rechler & Clemmons 1998, Bunn & Fowlkes 2003). One of these proteases was identified as pregnancy-associated plasma protein-A (PAPP-A) (Lawrence *et al.* 1999). PAPP-A has been shown to cleave inhibitory IGFBP-4 and consequently increase the IGF available for receptor activation in various cell systems *in vitro* (Conover *et al.* 1995, Byun *et al.* 2001, Ortiz *et al.* 2003). *In vivo*, intact IGFBP-4 is

postulated to form a local reservoir of IGFs that can be made available at critical times through regulated expression of PAPP-A and its associated proteolytic activity (Pintar *et al.* 1998). Recently, we generated PAPP-A-null mice which, similar to IGF-II-null mice, were born as proportional dwarfs (Conover *et al.* 2004). The loss of PAPP-A resulted in complete loss of IGFBP-4 proteolytic activity, with no alterations in embryonic *IgfII* or *Igfbp4* gene expression. The impact of the mutation occurred early in embryo development, prior to organogenesis, affecting skeletal ossification and overall body size. *In vitro* studies with embryonic fibroblasts derived from these animals supported a role for PAPP-A in regulating IGF-II bioavailability through IGFBP-4 proteolysis (Conover *et al.* 2004). We reasoned that if the PAPP-A-null phenotype was due to decreased IGF-II bioavailability during embryogenesis, then an increase in IGF-II to an extent that would overcome the inhibitory capacity of IGFBP-4 would rescue the growth retardation resulting from the absence of PAPP-A proteolytic activity.

One way an increase in the amount of IGF-II in mouse embryos could be achieved is through loss of type II

IGF/mannose-6-phosphate (M6P) receptors. Unlike the type I IGF and insulin signaling receptors, the type II IGF/M6P receptor functions to control extracellular IGF-II concentrations by receptor-mediated endocytosis and subsequent degradation of the growth factor in lysosomes (Braulke 1999). Mice lacking type II IGF/M6P receptors have markedly elevated IGF-II concentrations and exhibit fetal overgrowth due to overstimulation of type I IGF and insulin receptors (Ludwig *et al.* 1996). However, there are additional severe abnormalities in type II IGF/M6P receptor-null mice, including cardiac defects and edema, and these mice usually die pre- or peri-natally (Lau *et al.* 1994). Furthermore, the type II IGF/M6P receptor binds other ligands besides IGF-II, such as M6P-containing lysosomal enzymes and retinoic acid, and has additional transport functions (Gosh *et al.* 2003). To avoid those potentially confounding aspects of the type II IGF/M6P receptor-null mouse model, we chose an alternate approach to increased IGF-II during embryogenesis using $\Delta H19$ mutant mice.

In mice, the imprinted *IgfII* gene is located upstream and linked closely to the reciprocally imprinted *H19* locus on chromosome 7 (Zemel *et al.* 1992, Leighton *et al.* 1995a,b). This linkage between *IgfII* and *H19* and their imprinted features are conserved in humans (Zemel *et al.* 1992). The imprinted *H19* gene encodes an untranslated RNA (Brannan *et al.* 1990, Leighton *et al.* 1995a,b). According to the Enhancer Competition model, promoters for *H19* and *IgfII* share enhancers that are expressed downstream of *H19* (Bartolomei *et al.* 1993, Leighton *et al.* 1995b). Paternal-specific DNA methylation around the *H19* gene promoter functions to prevent the interaction of the paternal *H19* gene with enhancers, thereby making them available to engage the paternal *IgfII* gene. On the maternal allele, there is a promoter block in *H19* which prevents access to the *IgfII* promoter by the enhancer elements and, thus, *IgfII* expression is silenced. Imprinting of the *IgfII* gene can be disrupted by maternal inheritance of a targeted deletion of the *H19* gene and its 5' flanking region (Leighton *et al.* 1995a, Eggenschwiler *et al.* 1997). Thus, with maternally derived $\Delta H19$, the normally silent *IgfII* allele becomes transcriptionally active and the increased amount of IGF-II mRNA from both alleles yields a higher-than-normal level of translation product resulting in increased birth weight (Eggenschwiler *et al.* 1997). Mice inheriting the *H19* mutation from their mothers are 20–30% larger by weight than wild-type mice or mice inheriting the mutation from their fathers, and they are viable (Leighton *et al.* 1995a).

Thus, to test the hypothesis that an increase in IGF-II during embryogenesis is sufficient to rescue the dwarf phenotype of PAPP-A mutant mice, we generated *PAPP-A*/ $\Delta H19$ double mutants and characterized their phenotype in terms of body size and skeletal development in comparison with single mutants and control littermates.

These studies are critical *in vivo* correlates to *in vitro* studies defining the regulation and action of PAPP-A.

Materials and Methods

Mice

The strains of mice used in this study, which carry targeted mutations in the *PAPP-A* and *H19* loci, have been characterized previously (Leighton *et al.* 1995a, Eggenschwiler *et al.* 1997, Conover *et al.* 2004). Details of the genetic crosses to generate double mutants are described in the text. These studies were approved by Mayo's Institutional Animal Care and Use Committee.

Genotyping

H19 and *PAPP-A* mutations were detected by Southern analyses as described (Leighton *et al.* 1995a, Conover *et al.* 2004). Briefly, for $\Delta H19$, genomic tail tip DNAs were digested with *PstI* and hybridized to a ^{32}P -labeled probe which detected 6.6 kb and 3.3 kb fragments corresponding to the wild-type and mutant alleles respectively. For *PAPP-A*, DNAs were digested with *BamHI* and hybridized to a ^{32}P -labeled probe that detected 15 kb and 2.6 kb fragments corresponding to wild-type and mutant alleles respectively. Since the 15 kb fragment was not always clearly identified, *PAPP-A* gene-deletion was confirmed by PCR performed using primers 5'ATG ATT CAT GAG ATT GGG CAT AG 3' and 5'TGT TGT AAG GAG TGT TGA AGA AGC 3' to detect exon 4 in the mouse *PAPP-A* gene (Conover *et al.* 2004).

PCR-based sexing of embryos and newborn mice was performed according to the method of McClive and Sinclair (2001), using primer pairs for *Sry*, the master sex determining gene on the Y chromosome, and *myogenin*, a control gene.

Skeletal staining

Embryos were eviscerated, skinned, fixed in ethanol and then stained with Alcian Blue 8 GS (cartilaginous elements) and Alizarin Red S (mineralized elements), as adapted from McLeod (1980). The tissues were cleared with 1% KOH, and the skeletons stored in glycerol. Images were captured using a Nikon SMZ800 microscope, DXM1200F digital camera and ACT-1 software (Nikon).

Results

Genetic crosses

Mice receiving the $\Delta H19$ mutation from the maternal allele ($\Delta H19(\text{m-}/+)$) have approximately twofold increased levels of IGF-II in the embryo, due to expression

Table 1 Intercrosses between $\Delta H19(m-/+)$ /PAPP-A(+/-) females and PAPP-A(-/-) males

Designation	Genotype		Number of embryos	Observed frequency (%)	Expected frequency (%)	Male:Female
	H19	PAPP-A				
Control	+/+	+/-	32	24	25	13:19
$\Delta H19$ mutant	m-/+	+/-	31	24	25	14:17
PAPP-A-null	+/+	-/-	37	28	25	19:18
Double mutant	m-/+	-/-	31	24	25	17:14
Total			131	100	100	63:68

m-/+ : maternal inheritance of mutation.

from both the maternal and paternal alleles, with consequent fetal overgrowth (Leighton *et al.* 1995a, Eggenschwiler *et al.* 1997). In contrast, paternal transmission of the mutation ($\Delta H19(+/-)$) has no phenotypic consequences for the progeny. The *PAPP-A* gene is not imprinted and both males and females with homozygous deletion are 60–70% of the body weight of wild-type littermates (Conover *et al.* 2004). Accordingly, paternally transmitted female H19 heterozygotes ($\Delta H19(+/-)$) were first crossed with PAPP-A(-/-) males. Of these offspring, females inheriting the H19 mutant allele ($\Delta H19(m-/+)$) and heterozygous for *PAPP-A* deletion were again mated to male PAPP-A(-/-) mice (4 breeding cages; 2 females:1 male) to generate embryos belonging to four genotypic classes (Table 1). Table 1 also shows that the expected Mendelian distribution was observed; therefore, there was no evidence for a genotype effect on viability. Furthermore, there did not appear to be gender bias in the genotypes.

As shown in Fig. 1, deletion of the *H19* gene in maternal heterozygotes resulted in a 26% increase in body mass at birth (1.92 g vs 1.52 g), similar to what has been reported previously (Leighton *et al.* 1995a, Eggenschwiler *et al.* 1997). PAPP-A-null mice from these crosses had body weights at birth that were 74% that of mice heterozygous for the *PAPP-A* mutation and negative for the *H19* mutation (1.12 g vs 1.52 g). We used this latter genotype as our control group since, in previous studies, there was no significant difference in body weights between PAPP-A heterozygous and wild-type mice (Conover *et al.* 2004). Furthermore, additional breeding cages of $\Delta H19(m-/+)$ /PAPP-A(+/-) and PAPP-A(+/-) mice were set up and the newborn weights of PAPP-A(+/+) and PAPP-A(+/-) mice that were $\Delta H19$ -negative were similar (1.52 $\pm$ 0.068 g and 1.55 $\pm$ 0.027 g respectively). The reason for differences in absolute birth weights of the PAPP-A(-/-) mice from these crosses and from the previous study (Conover *et al.* 2004) is unclear, but may be due to the mixed C57Bl/6 backgrounds of the mice in the current study. Importantly, mice that were mutant for both genes were

indistinguishable in size from control animals in their birth weights (Fig. 1) and postnatal growth rates (Fig. 2). These phenotype differences were similarly observed if the data were analyzed separately by gender (data not shown).

Skeletal staining

Embryonic day (E) 16.5 embryos from 4 litters (45 embryos) of $\Delta H19(m-/+)$ mutant and PAPP-A(+/-) crosses were stained for bone and cartilage to determine whether the delay in ossification observed in PAPP-A-null embryos (Conover *et al.* 2004) could be prevented with increased IGF-II. Genotype and gender were determined for all embryos. Representative embryos are presented in Fig. 3. Overall size differences are evident at E16.5. Thus, $\Delta H19$ mutants were larger than controls, and PAPP-A-null embryos were smaller than controls. Double mutants were approximately the same size as the control embryos. In general, skeletal ossification appeared to be similar in

Figure 1 Birth weights of progeny from intercrosses between $\Delta H19(m-/+)$ /PAPP-A(+/-) females and PAPP-A(-/-) males. Results are means $\pm$ S.E.M. for the genotypes presented in Table 1. * $P < 0.001$, significantly different from control.

Figure 2 Postnatal growth curves of $\Delta H19$ mutant (■), control (○), PAPP-A(-/-) (▲) and double mutant (●) mice. Values are means $\pm$ S.E.M. of 10–30 individual weights.

$\Delta H19$ mutants and controls. At this stage of development, there is substantial mineralization of bones that form both via endochondral ossification (vertebrae, peripheral phalanges) and intramembranous ossification (cranial vault) (Ohya et al. 1997). We generally saw mineralization of 3 to 4 digits, 3 to 4 caudal vertebrae and the frontal, parietal, and interparietal bones of $\Delta H19$ mutants and controls. On the other hand, PAPP-A-null mice negative for $\Delta H19$ mutation showed reduced ossification of the caudal vertebrae, terminal phalanges, and cranial vault (especially the parietal and interparietal bones), as was reported previously (Conover et al. 2004). Embryos with double mutations exhibited skeletal ossification patterns similar to controls.

Discussion

In this study, we demonstrate that rescue of the growth retardation and delayed ossification that occurs during fetal development in PAPP-A-null mice is associated with increased IGF-II expression during embryogenesis. This was achieved through relaxation of *IgfII* imprinting via a mutation in the reciprocally imprinted *H19*. These data support a critical function of the IGFBP protease, PAPP-A, in regulating IGF bioavailability *in vivo*.

Imprinting signals disrupted by the removal of the *H19* promoter from the maternal chromosome leads to the activation of the maternal *IgfII* gene. This is reflected in approximately twofold increased levels of IGF-II in the embryo due to expression from both the maternal and paternal alleles (Leighton et al. 1995a, Eggenschwiler et al. 1997). Thus, somatic overgrowth of $\Delta H19$ heterozygotes for the maternal deletion is attributed to the increase in

IGF-II. In this study, inheritance of the $\Delta H19(m-/+)$ mutation and the consequent increase in IGF-II expression rescued the dwarf phenotype of PAPP-A-null mice. This underscores the crucial role of dose regulation of IGF-II, through imprinting and regulated expression of PAPP-A, to the developing embryo. Both growth retardation of PAPP-A-null mice and overgrowth of $\Delta H19$ maternal heterozygotes occurred exclusively during embryogenesis, whereas after birth both classes of mutants grew at the same rate as control mice. These findings fit with the model that IGF-II is critical during early embryogenesis for determining optimal body size (Baker et al. 1993, Leighton et al. 1995a, Eggenschwiler et al. 1997, Louvi et al. 1997, Burns & Hassan 2001). We now add a new dimension to this model by implicating PAPP-A as a key regulator of IGF-II bioavailability during fetal development. Although these, along with our previous *in vitro* data (Conover et al. 2004), are supportive of the relationship between PAPP-A and IGF-II in fetal development, they do not establish a causal relationship and they do not rule out an effect of PAPP-A on IGF-I bioavailability during later embryogenesis (Baker et al. 1993). Furthermore, these studies do not address the role of IGFBP-4 directly or the possible involvement of other IGFBPs in this model. Refinement of the model will require further study.

The skeletal findings indicate that elevated IGF-II resulting from the $\Delta H19$ mutation was also associated with rescue of the ossification defects in PAPP-A-null mice. IGF-II has a significant role in embryonic skeletogenesis. In addition to its mitogenic activity, IGF-II promotes differentiation of cells that are primarily of mesodermal origin (Milner & Hill 1984). *IgfII* is strongly expressed in head mesoderm, and in the cephalic portion of neural crest cells, somites forming the axial skeleton, and lateral mesoderm forming limb buds (Stylianopoulou et al. 1988, Lee et al. 1990). The pattern of *IgfII* gene expression during embryogenesis overlaps significantly with *PAPP-A*, which is also expressed in head mesenchyme and neural crest cells, somites and limbs (Stylianopoulou et al. 1988, Lee et al. 1990, Conover et al. 2004). Importantly, there is a striking association of *IgfII* and *Igfbp4* gene expression (Cerro et al. 1993, Schuller et al. 1993), thereby allowing establishment of an IGF-II/IGFBP-4 reservoir that could be rapidly accessed by regulated increases in PAPP-A expression. Besides the proposed roles in embryological development, the PAPP-A/IGFBP-4/IGF system may be involved in post-natal bone formation as well, since PAPP-A and IGFBP-4 are highly expressed by osteoblasts and have been shown to regulate IGF action in these cells *in vitro* (Wang et al. 1995, van Kleffens et al. 1998, Ortiz et al. 2003). Furthermore, the importance of regulation of local IGF action in adult bone by PAPP-A is suggested by the finding of defective fracture repair in PAPP-A-null mice (Miller et al. 2004).

In conclusion, these data provide strong genetic evidence that PAPP-A plays a unique role pre-natally that

Figure 3 Skeletal development of embryonic day 16.5 embryos from (A) control, (B) Δ H19 mutant, (C) PAPP-A-null and (D) double mutant littermates is shown. The associated lower panels (E-H) show close-up images of the forelimbs of these embryos. Red staining identifies mineralized elements. Blue staining identifies cartilaginous elements. f, frontal; p, parietal; i, interparietal.

involves the optimization of specific IGF-II action. This is a new concept critical to our understanding of the IGF system *in vivo*. Furthermore, these findings implicate a similar role post-natally in IGF-mediated wound healing, bone remodeling, and vascular repair (Bayes-Genis *et al.* 2001, Chen *et al.* 2003, Miller *et al.* 2004), and perhaps other as yet undetermined events in which locally regulated IGF bioavailability has physiologic and pathophysiologic importance.

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