Metalloproteinase pregnancy-associated plasma protein A is a critical growth regulatory factor during fetal development

Cheryl A. Conover[1,*], Laurie K. Bale[1], Michael T. Overgaard[2], Edward W. Johnstone[1], Ulla H. Laursen[2], Ernst-Martin Füchtbauer[2], Claus Oxvig[2] and Jan van Deursen[3]

[1]The Division of Endocrinology, Metabolism and Nutrition, Endocrine Research Unit, Mayo Clinic and Mayo Foundation, 200 First Street SW, Rochester, MN 55905, USA
[2]The University of Aarhus, Department of Molecular Biology, Science Park, Gustav Wieds Vej 10C, DK-8000, Aarhus C, Denmark
[3]The Department of Pediatric and Adolescent Medicine, and Department of Biochemistry and Molecular Biology, Mayo Clinic and Mayo Foundation, 200 First Street SW, Rochester, MN 55905, USA
*Author for correspondence (e-mail: conover.cheryl@mayo.edu)

Accepted 20 November 2003

Development 131, 1187-1194
Published by The Company of Biologists 2004
doi:10.1242/dev.00997

Summary

Pregnancy-associated plasma protein A (PAPPA) is a metzincin superfamily metalloproteinase in the insulin-like growth factor (IGF) system. PAPPA increases IGF bioavailability and mitogenic effectiveness in vitro through regulated cleavage of IGF-binding protein 4 (IGFBP4). To determine its function in vivo, we generated PAPPA-null mice by gene targeting. Mice homozygous for targeted disruption of the *PAPPA* gene were viable but 60% the size of wild-type littermates at birth. The impact of the mutation was exerted during the early embryonic period prior to organogenesis, resulting in proportional dwarfism. *PAPPA*, *IGF2* and *IGFBP4* transcripts co-localized in wild-type embryos, and expression of *IGF2* and *IGFBP4* mRNA was not altered in PAPPA-deficient embryos. However, IGFBP4 proteolytic activity was completely lacking in fibroblasts derived from PAPPA-deficient embryos, and IGFBP4 effectively inhibited IGF-stimulated mitogenesis in these cells. These results provide the first direct evidence that PAPPA is an essential growth regulatory factor in vivo, and suggest a novel mechanism for regulated IGF bioavailability during early fetal development.

Key words: Pregnancy associated plasma protein A, Insulin-like growth factor, Gene targeting, Metalloproteinase

Introduction

The insulin-like growth factors (IGF1 and IGF2) are important determinants of fetal growth and postnatal development (Baker et al., 1993; Stewart and Rotwein, 1996). IGF bioactivity is modulated by a family of six IGF-binding proteins (IGFBPs) (Firth et al., 2002), the structure and function of which can be regulated by specific IGFBP proteases (Wetterau et al., 1999; Bunn and Fowlkes, 2003). Recently, pregnancy-associated plasma protein A (PAPPA) was identified as a novel zinc-binding metalloproteinase secreted by normal human fibroblasts with IGFBP4 as its substrate (Lawrence et al., 1999). IGFBP4 is an inhibitor of IGF action and, in this capacity, may serve as a pericellular reservoir for IGFs (Mohan et al., 1989; Pintar et al., 1998). Cleavage of IGFBP4 by PAPPA results in increased bioavailability and mitogenic effectiveness of IGFs in vitro (Conover et al., 1995; Byun et al., 2001; Ortiz et al., 2003). Along with fibroblasts, PAPPA proteolytic activity has been identified in cultured osteoblasts (Conover et al., 1995; Qin et al., 2000; Ortiz et al., 2003), vascular smooth muscle cells (Bayes-Genis et al., 2001a) and ovarian granulosa cells (Conover et al., 2001). Furthermore, increased PAPPA expression in vivo has been shown to be associated with conditions of heightened IGF activity, such as neointimal hyperplasia following balloon angioplasty of pig coronary arteries (Bayes-Genis et al., 2001a), active atherosclerotic plaques in human coronary arteries (Bayes-Genis et al., 2001b), and healing human skin (Chen et al., 2003). Although PAPPA was originally described as a protein of placental origin circulating in human pregnancy (Lin et al., 1974), these data indicate additional roles for PAPPA, outside of pregnancy, in localized and finely controlled growth states. However, direct experimental evidence to date has been lacking.

Human PAPPA has an elongated zinc-binding motif, with residues coordinating the catalytic zinc ion of the active site, and a structurally important methionine residue located downstream in the so-called Met-turn, both of which are strictly conserved within the metzincin superfamily of metalloproteinases (Stocker et al., 1995; Boldt et al., 2001; Overgaard et al., 2003). Metzincins are remarkably similar in their tertiary structure, although they have only limited sequence identity. PAPPA is distinct from the other four metzincin groups (astacins, serralysins, adamalycins or reprolysins, and matrix metalloproteinases) because of a characteristic residue directly following the zinc-binding motif, and the unusual distance between the zinc-binding motif and the Met-turn (Boldt et al., 2001). The overall sequence identity between murine and human PAPPA is 91% (Soe et al., 2002), with the coding of all residues of the zinc binding and Met-turn consensus conserved in exon 4 (Overgaard et al., 2003).

In this study we generated PAPPA-null mice by gene targeting and demonstrate a crucial role for PAPPA during fetal development.

Materials and methods

Construction of replacement vector

To construct a vector for targeting of the *PAPPA* gene, we first screened a 129 mouse embryonic stem (ES) cell genomic library (using Lambda FIX II, Stratagene, LaJolla, CA), with human *PAPPA* cDNA (Overgaard et al., 2000), and isolated a phage λ clone carrying DNA that included exon 4 of the mouse *PAPPA* gene, which encodes the protease domain (Overgaard et al., 2003). Fragments of the cloned *PAPPA* gene and a pKO Scrambler Series vector (Stratagene) were used for construction of the replacement vector. The neomycin-resistant gene (*neo*) cassette, replacing 1.6 kb of *PAPPA* gene sequence, including most of exon 4, was flanked by a 6 kb *Pst*I (P) fragment and a 2 kb *Nsi*I (N) fragment of mouse PAPPA locus DNA (5′ and 3′, respectively). Addition of *neo* also introduced a novel *Bam*HI (B) restriction site. A cassette for *thymidine kinase* gene selection was located upstream of the first set of polylinker restriction sites. A schematic of the vector and targeting strategy is shown in Fig. 1A.

Generation of PAPPA-null mice by homologous recombination

Linearized replacement vector DNA was introduced into 129-derived ES cells by electroporation (BioRad Gene Pulsar at 230 V, 500 uF capacitance), and the cells seeded and selected on feeder layers of irradiated fibroblasts. The *neo* and *thymidine kinase* gene markers in the vector allowed the application of a positive-negative selection protocol in the presence of the drugs, G418 and FIAU. Drug-resistant colonies were picked and expanded (without selection) for further analysis. Seven prospective ES clones with targeted *PAPPA* alleles were identified by Southern blot analysis of ES cell DNA. Four of these seven independent targeted clones were microinjected into C576Bl/6 blastocysts and transferred into the uterine horn of foster mothers to generate chimeric mice, scored by presence of agouti coat hair. The frequency of formation of overt chimeria was high (>50%) for three of the four strains of blastocysts.

Male chimeras from these three clones (designated E3, E7, D10) were then cross-bred with C57Bl/6 females and germ-line transmission was obtained for all three. Heterozygous mutants were identified by Southern analysis of tail tip DNA. After transmission of the mutations, intercrosses between heterozygous progeny yielded homozygous mutants for E3, E7 and D10. Littermates obtained by breeding heterozygous males and females were used for all phenotypic analyses.

Genotyping

Southern analysis

ES cells and mouse tail tip DNA were digested with *Bam*HI, run on a 0.8% agarose gel and transferred to Hybond (Amersham Pharmacia, Arlington Heights, IL). Membranes were prehybridized for 1 hour at 65°C in RapidHyb and then hybridized overnight at 65°C in the same solution containing ^{32}P-labeled 3′ probe (see Fig. 1A). Membranes were washed at 65°C in 1×SSC/0.1% SDS, 0.3×SSC/0.1% SDS and 0.1×SSC/0.1% SDS, and then exposed to film. With this probe, homologous recombination in ES cells would be expected to show both the wild-type 15 kb fragment and a mutant 2.6 kb fragment of *Bam*HI-digested DNA. For mouse tail DNA, we would expect wild-type mice to have a single 15 kb fragment, heterozygous mice to have both 15 kb and 2.6 kb fragments, and homozygous mutants to have single 2.6 kb fragments (see insert Fig. 1A).

PCR

PCR on mouse tail DNA was 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 *PAPPA* gene; and 5′-AGG ATC TCC TGT CAT CTC ACC TTG CTC CTG-3′ and 5′-AAG AAC TCG TCA AGA AGG CGA TAG AAG GCG-3′, to detect *neo*. PCR reactions containing these primers generated fragments in ethidium bromide-stained agarose gels of 223 bp for the endogenous exon 4-containing *PAPPA* gene and 492 kb for the recombinant *neo*-containing gene.

PCR-based sexing of mouse embryos was performed according to the method of McClive and Sinclair (McClive and Sinclair, 2001), using yolk sac DNA and primer pairs for *Sry*, the master sex determining gene on the Y chromosome, and *myogenin*, a control gene. It is known that there is a great deal of variability in embryo sizes even among littermates, and that male embryos may develop faster than females. Therefore, yolk sacs from embryos were sexed by PCR to rule out possible gender bias.

RT-PCR

Total RNA was extracted from whole embryos and tissues using RNeasy Mini kit (Qiagen, Valencia, CA) and treated with DNase (DNA-free, Ambion, Austin, TX). RNA (400 ng) was reverse-transcribed using TaqMan Reverse Transcription reagents (PE Biosystems, Foster City, CA), according to manufacturer's instructions. Primer sequences for assessment of *PAPPA* mRNA expression were as above for a predicted PCR product of 223 bp. Having established the linear range, amplifications were performed for 32 cycles. The initial denaturation was performed at 94°C for 5 minutes, cycles were at 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 1 minute, and full-length products were obtained by a final elongation period of 10 minutes at 72°C. PCR reaction products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Primary cell cultures

Primary cultures of mouse embryo fibroblasts (MEF) were derived from E13.5 day embryos from heterozygous matings. Tissue from each embryo was used for genotyping. Embryos were washed, minced, trypsinized and single cell suspensions plated in high glucose DMEM, containing glutamine, penicillin, streptomycin, β-mercaptoethanol and 10% ES cell-tested FCS. Cells at passage 2-4 were used for experiments.

IGFBP4 protease activity assay

Primary cultures of MEF were washed and changed to serum-free medium. After 24 hours, conditioned medium was collected for cell-free assay. IGFBP4 proteolysis was assayed as described previously (Conover et al., 1995; Conover et al., 2001; Lawrence et al., 1999; Overgaard et al., 2000), by incubating MEF-conditioned medium samples at 37°C for 6 hours with ^{125}I-IGFBP4 in the absence and presence of 5 nM IGF2. Proteins were separated by SDS-PAGE and visualized by autoradiography.

Cell proliferation

[^{3}H]Thymidine incorporation was performed as described previously (Conover et al., 1995; Ortiz et al., 2003). MEF cultures were grown to 80% confluence, washed twice and changed to 0.1% FCS for 48 hours prior to experimental additions. [methyl-^{3}H]Thymidine (0.5 μCi/ml; DuPont-NEN, Boston, MA) was added for 22-26 hours after the experimental additions. For the experiments in Table 2, cultures were washed three times immediately before addition of IGFs. For the experiments in Fig. 5B, 25 nM IGFBP4 or IGFBP3±5 nM IGF were directly added to the 48-hour-conditioned medium. Results are calculated as the percentage of total counts in the incubation medium that are incorporated into acid-precipitable material.

Receptor phosphorylation

MEF cultures were washed and changed to 0.1% FCS for 48 hours. Twenty-five nM recombinant wild-type or protease-resistant IGFBP4 (Overgaard et al., 2000; Ortiz et al., 2003) was added and incubation continued for an additional hour. Cultures were then washed with ice-cold PBS containing 2 mM vanadate and solubilized in lysis buffer

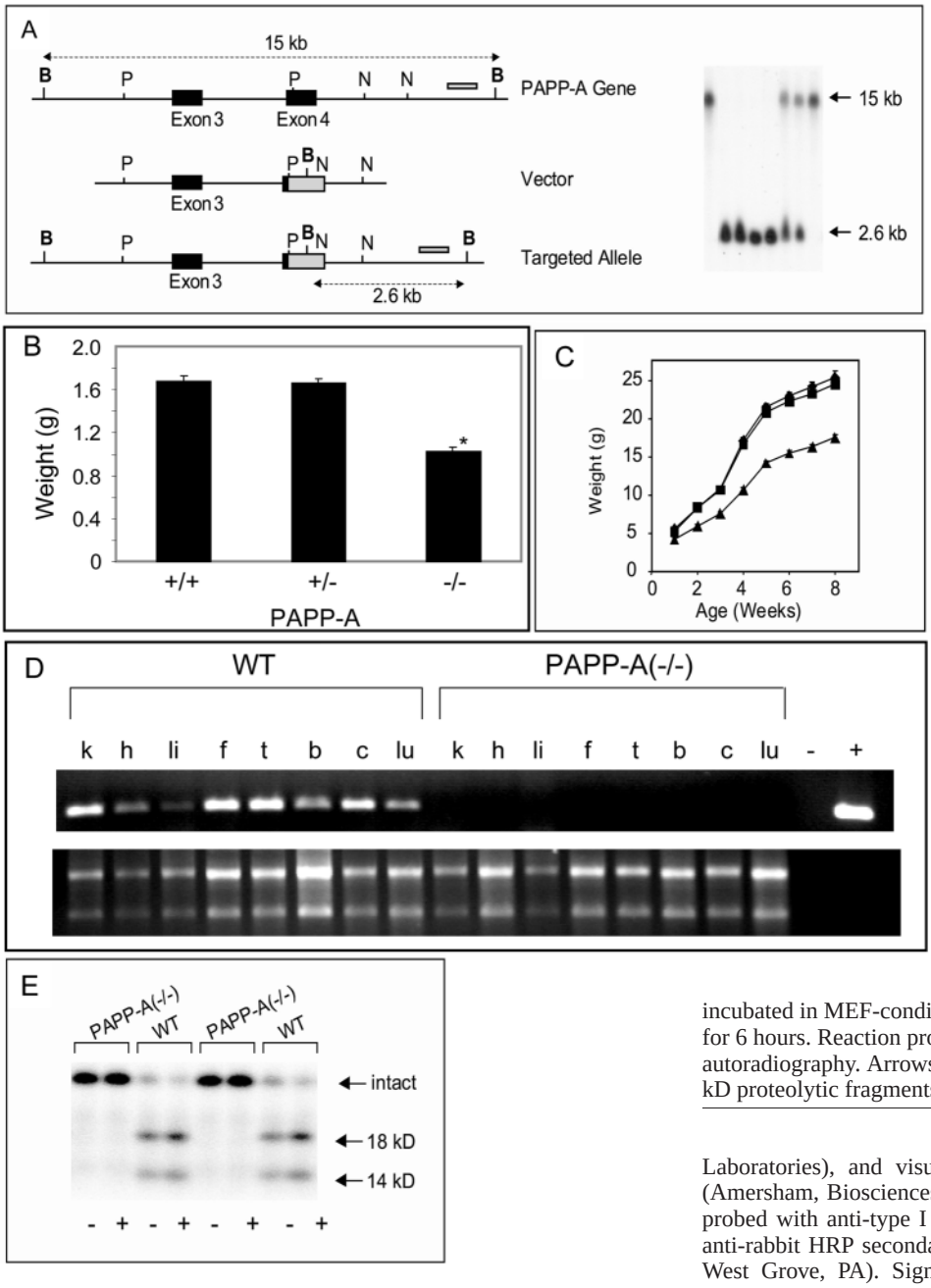

Fig. 1. Generation of PAPPA-null mice. (A) Schematic representation of the mouse gene in the region of exons 3 and 4 of the *PAPPA* locus, the replacement vector and the targeted allele. The position of the probe used for Southern analysis is indicated by the dark gray bar, and the sizes of the endogenous and targeted *Bam*HI (B) genomic DNA fragments recognized by this probe are shown. An example of genotyping of mouse tail DNA is shown in the insert. Wild-type mice have a single 15 kb band (lanes 1, 8), heterozygous mice have both 15 kb and 2.6 kb bands (lanes 6, 7), and homozygous mutants have a single 2.6 kb band (lanes 2-5). (B) Weights of wild-type (+/+), heterozygous (+/–) and PAPPA-deficient (–/–) mice at birth. Results are mean±s.e.m.; n=20 for each genotype. *, significantly different from wild-type, P<0.0001. (C) Growth curves of wild-type (◆), heterozygous (■) and homozygous (▲) PAPPA$^{-/-}$ mutant mice. Values are mean±s.e.m. of 28-141 individual weights. (D) RT-PCR for *PAPPA* mRNA expression (upper panel) in tissues from wild-type (WT) and PAPPA$^{-/-}$ mice. k, kidney; h, heart; li, liver; f, femur; t, tibia; b, brain; c, calvaria; lu, lung. –, negative control; +, positive control. Lower panel shows ethidium bromide staining of tissue 28S and 18S RNA to validate integrity and loading. (E) IGFBP4 proteolysis in medium conditioned by embryonic fibroblasts derived from wild-type (WT) and PAPPA$^{-/-}$ mice. ^{125}I-IGFBP4 was incubated in MEF-conditioned medium without (–) or with (+) IGF2 for 6 hours. Reaction products were analyzed by SDS-PAGE and autoradiography. Arrows indicate intact IGFBP4 and 18 kD and 14 kD proteolytic fragments.

[20 mM Tris (pH 7.6), 137 mM NaCl, 2 mM sodium orthovanadate, 1% NP40, 10% glycerol, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 40 mM β-glycerolphosphate, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin]. The lysed material was precleared with protein A-Sepharose (Oncogene Research Products, Boston, MA) and non-specific rabbit IgG before overnight incubation at 4°C with 4 μg/ml antibody to type I IGF receptor β-subunit (C-20, Santa Cruz, Biotechnology, Santa Cruz, CA) or non-specific rabbit IgG. The immune complexes were precipitated by incubation with protein A-Sepharose for 6 hours at 4°C, followed by centrifugation and four washes with PBS containing Triton X-100, sodium deoxycholate and SDS. The proteins were separated by SDS-PAGE under reducing conditions, transferred to PVDF membrane, probed with a 1:1000 dilution of anti-phosphotyrosine antibody (PY20, Transduction Laboratories, Lexington, KY) and a 1:2000 dilution of secondary antibody (goat anti-mouse HRP, Transduction Laboratories), and visualized using enhanced chemiluminescence (Amersham, Biosciences, Piscataway, NJ). The membranes were re-probed with anti-type I IGF receptor antibody (1 μg/ml), with goat anti-rabbit HRP secondary (Johnson ImmunoResearch Laboratories, West Grove, PA). Signal intensities were quantified by scanning densitometry (UltraScan XL laser densitometer, Pharmacia LKB Biotechnology, Piscataway, NJ).

Skeletal staining

Embryos were eviscerated, skinned, fixed in ethanol and then stained with Alcian Blue 8GS (cartilaginous elements) and Alizarin Red S (mineralized elements) at 37°C for 3-5 days, as adapted from McLeod (McLeod, 1980). The tissues were cleared with 1% KOH and the skeletons stored in glycerol.

In situ hybridization

Whole-mount embryos were hybridized with digoxigenin-labeled RNA probes as described previously (Fuchtbauer et al., 1995). cDNA clones, all contained in vectors with the T7 promoter, were used as templates for in vitro transcription. Murine PAPPA clone E11 (Soe et al., 2002) was used for PAPPA. Rat *IGF2* and mouse *IGFBP4* cDNAs were kindly provided by Dr C. Bondy (NIH-NICHD, Bethesda, MD) and Dr S. Drop (Rotterdam, The Netherlands), respectively.

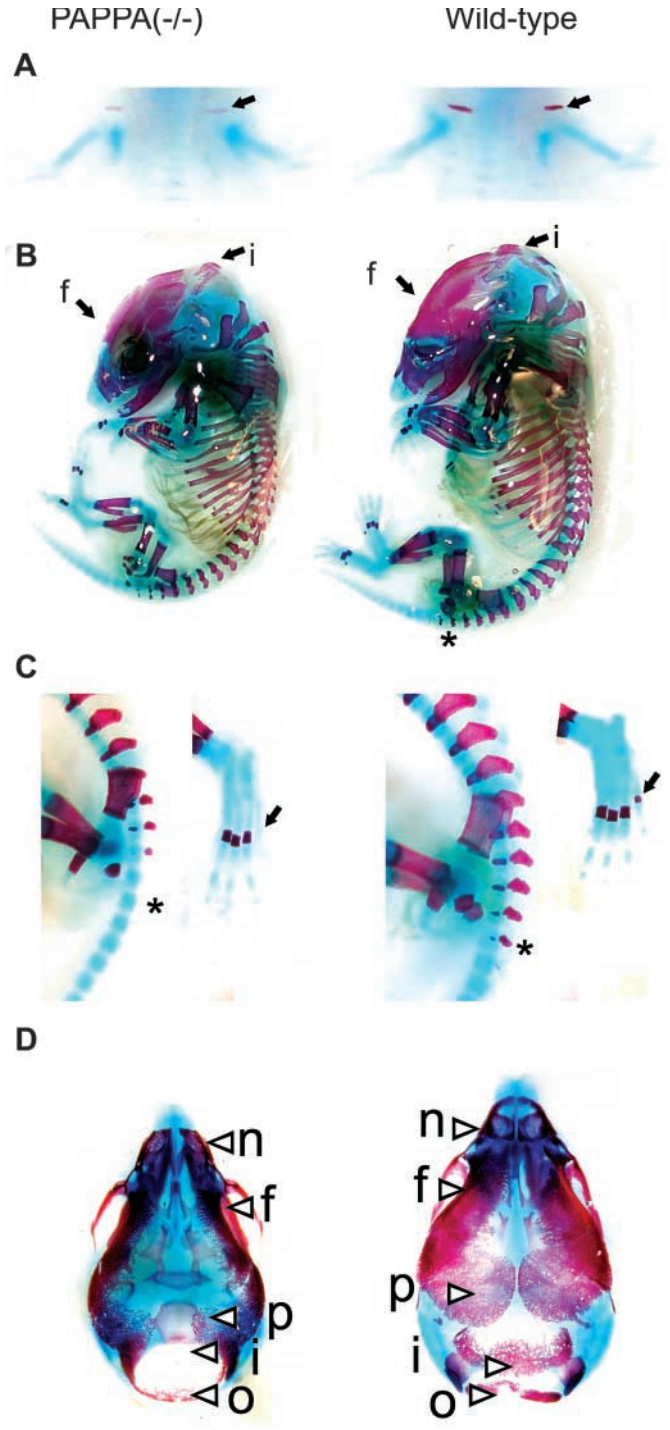

Fig. 2. Skeletal development. (A) At E13.5, mineralization of the clavicle (arrows) is delayed in the PAPPA$^{-/-}$ compared with wild-type mice. (B) At E16.5, no caudal vertebrae were mineralized in the PAPPA$^{-/-}$ mouse, whereas three to four caudal vertebral elements were undergoing mineralization in wild-type littermates (asterisk). The frontal (f) and interparietal (i) bones of the cranial vault are both undergoing mineralization at E16.5, but these processes are far less complete in PAPPA$^{-/-}$ embryos. (C) Close-up of digits (arrow) and vertebrae (asterisk). Note an additional metatarsal had initiated mineralization in the wild-type compared with the PAPPA$^{-/-}$ mice (arrow). (D) Close-up of cranial vault. The nasal (n), parietal (p) and occipital (o) bones are also delayed.

Results

Generation of PAPPA-null mice

The general scheme for the generation of PAPPA-null mice through homologous recombination in embryonic stem cells is presented in Fig. 1A. Interbreeding of heterozygous mice gave rise to the expected mendelian distribution of the *PAPPA* gene. The male:female ratio for homozygous mutants was approximately 50:50. Homozygous PAPPA-deficient mice were viable, but only 60% of the size of their wild-type littermates at birth (Fig. 1B). They remained 40% smaller during postnatal development (Fig. 1C). This growth-deficient phenotype was the same in three independently targeted mouse lines, indicating a consistent effect of *PAPPA* deletion. There was no apparent size or growth difference between wild-type and heterozygous mice. Allometric measures of various major internal organs of 8-week-old mice indicated a general proportionate decrease in the wet weight of all organs, as well as in body and bone length in PAPPA$^{-/-}$ versus wild-type mice (Table 1). Apart from their small size, the PAPPA-deficient mice appeared normal and were fertile.

RNA prepared from neonatal tissues verified the complete loss of *PAPPA* expression by RT-PCR in homozygous mutants (Fig. 1D). Moreover, primary mouse embryo fibroblasts (MEF) derived from E13.5 wild-type and homozygous PAPPA mutant littermates were cultured and the conditioned media assayed for the presence of functional PAPPA, i.e. IGFBP4 protease activity. ^{125}I-IGFBP4 incubated with wild-type MEF conditioned medium was fully proteolyzed into fragments of 18 and 14 kDa (Fig. 1E), similar to the cleavage pattern produced by recombinant PAPPA (Overgaard et al., 2000). IGF2 is a cofactor for the proteolytic activity in vitro as a result of its ability to bind IGFBP4 and increase susceptibility to PAPPA (Byun et al., 2000; Laursen et al., 2001), and fetal mouse fibroblasts secrete high levels of IGF2 (Adams et al., 1983), sufficient to promote PAPPA-mediated IGFBP4 proteolysis in these cultures. By contrast, medium conditioned by MEF from PAPPA$^{-/-}$ embryos exhibited no proteolysis of ^{125}I-IGFBP4, either without or with addition of IGF2.

Skeletal development

Investigation of the effect of *PAPPA* gene disruption on skeletal development during embryogenesis indicated developmental delay in the appearance of ossification centers in the clavicle, facial and cranial bones, vertebrae, and the digits of the forelimbs and hindlimbs (Fig. 2). The initiation of mineralization occurs between E12.5 and E13.5 in the mouse. As expected (Huang et al., 1997), the first bone to mineralize in both wild-type and PAPPA$^{-/-}$ mice was the clavicle. At E13.5, the middle portion of the clavicle was clearly mineralized in the wild type, whereas mineralization was just initiated in PAPPA$^{-/-}$ littermates (Fig. 2A). The delay in mineralization occurred in bones that form via intramembranous ossification (cranial vault) and endochondral ossification (vertebrae, metatarsals) (Fig. 2B,C,D). At E16.5, no caudal vertebrae were mineralized in the PAPPA$^{-/-}$ mouse, whereas three to four caudal vertebral elements were undergoing mineralization in wild-type littermates. Likewise an additional metatarsal had initiated mineralization in the wild type compared with the PAPPA$^{-/-}$ mice. The frontal and

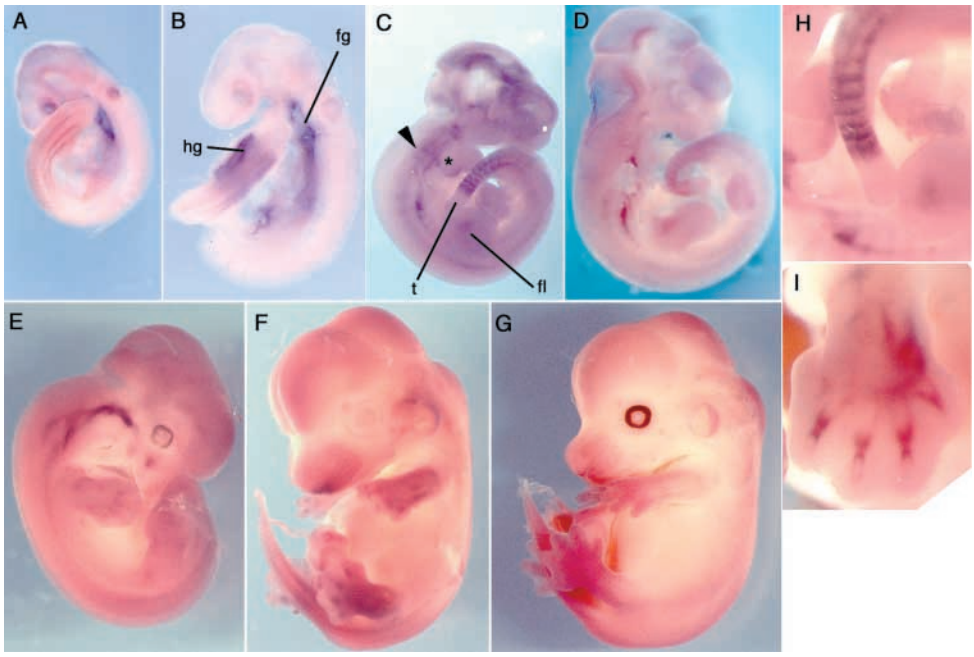

Fig. 3. In situ hybridization for PAPPA expresssion in wild-type mouse embryos. At (A) E8.5 and (B) E9.5, PAPPA expression is mainly found in the developing foregut (fg) and hindgut (hg). Staining in the optic and otic vesicle is an artifact. At (C) E10.5 and (D) E11.5, PAPPA expression is seen in the cells resembling migrating neural crest cells (arrowhead), in branchial arches (asterisk), in the forelimbs (fl) and in a segmented pattern in the tail (t). At (E) E12.5, (F) E13.5 and (G) E14.5, PAPPA expression persisted in the developing limbs, tail, nasal region and the developing outer ear. Note that in F an albino embryo is shown, indicating that there is no PAPPA expression in the developing eye. (H) Higher magnification of the tail at E10.5 to demonstrate the segmented expression in the developing somites. (I) Higher magnification of the forelimb of an E13.5 embryo showing PAPPA expression in the developing joints of the digits.

interparietal bones of the cranial vault were both undergoing mineralization at E16.5, but these processes were far less complete in PAPPA$^{-/-}$ mice. Examination of several skeletons from E13.5 to E18.5 littermates indicated ossification delays of approximately 1 day.

PAPPA expression during fetal development

Whole embryo in situ hybridization indicated the presence of *PAPPA* transcripts in wild-type mice at all embryonic stages investigated (Fig. 3). PAPPA was expressed in the paraxial mesoderm in the presomitic cells shortly before segmentation and in the posterior part of the somites, a pattern seen clearly from E9.5 to E13.5 (Fig. 3B-F,H). In the limbs, PAPPA expression was first detected in the forelimb at E10.5 (Fig. 3C) as a diffuse expression in the center of the limb bud. At E11.5 (Fig. 3D), there was a clear pattern in both forelimb and hindlimb. As the limb develops, the pattern in the limbs changes in a manner suggesting the expression to be in pre-cartilage cells. At E10.5 (Fig. 3C), defined PAPPA expression can be seen in the brain. The staining seen in the region behind the ear and on the side appeared to be neural crest cells. There was also expression of PAPPA on the distal part of the branchial arches.

Table 1. Allometric measurements

	Wild type	PAPPA$^{-/-}$	%	*P* value
Body weight (g)	33.7±1.5	18.0±0.9	53	<0.0001
Heart (g)	0.1760±0.0075	0.1070±0.0058	61	<0.0001
Spleen (g)	0.0930±0.0067	0.0400±0.0033	43	<0.0001
Liver (g)	1.674±0.093	0.922±0.084	55	<0.0001
Kidney (g)	0.208±0.008	0.121±0.009	58	<0.0001
Body length (mm)*	95.0±1.3	84.0±2.1	88	0.0012
Femur length (mm)	15.9±0.3	14.0±0.2	88	0.0004

Body and tissue weights and lengths (mean±s.e.m.) of six wild-type and six PAPPA$^{-/-}$ male mice at 8 weeks.

%, percent of PAPPA$^{-/-}$ weight or length compared with wild-type weight or length.

*Body length was measured as nose-to-anus.

PAPPA-deficiency affects IGF bioavailability but not expression

PAPPA expression in the mouse embryo parallels that of IGF2 (Stylianopoulou et al., 1988; Lee et al., 1990), the dominant IGF during early fetal development in rodents (Baker et al., 1993). *IGF2* mRNA was expressed in the head mesenchyme formed from both the mesoderm and cephalic portion of the neural crest, and in most mesoderm-derived tissues, particularly somites and lateral mesoderm (Fig. 4A,B,D,E). In addition, IGF2 expression in chondrocytes has been shown to be elevated prior to ossification (Stylianopoulou et al., 1988). There appeared to be no difference in the level or pattern of *IGF2* mRNA expression between wild-type and PAPPA$^{-/-}$ mice (Fig. 4A,B versus Fig. 4D,E). However, delays in limb and tail development were apparent in E12.5 PAPPA$^{-/-}$ embryos. Most mesodermally-derived tissues of wild-type and PAPPA$^{-/-}$ embryos also expressed *IGFBP4* mRNA (Fig. 4C,F). This pattern of IGFBP4 expression was consistent with previous studies (Schuller et al., 1993; Pintar et al., 1998). Similarly, northern analysis for *IGF2* and *IGFBP4* mRNA showed no difference of expression between wild-type and PAPPA-null mouse embryos (data not shown). Serum IGF1 levels were not significantly different between newborn wild-type mice (32±5, n=11) and PAPPA-deficient mice (35±4, n=13). Fibroblasts from PAPPA-null E13.5 embryos were as responsive as those from wild-type embryos to growth stimulation by IGF1 and IGF2, indicating that the capacity for type I IGF receptor signaling was not altered in cells of PAPPA-null mice (Table

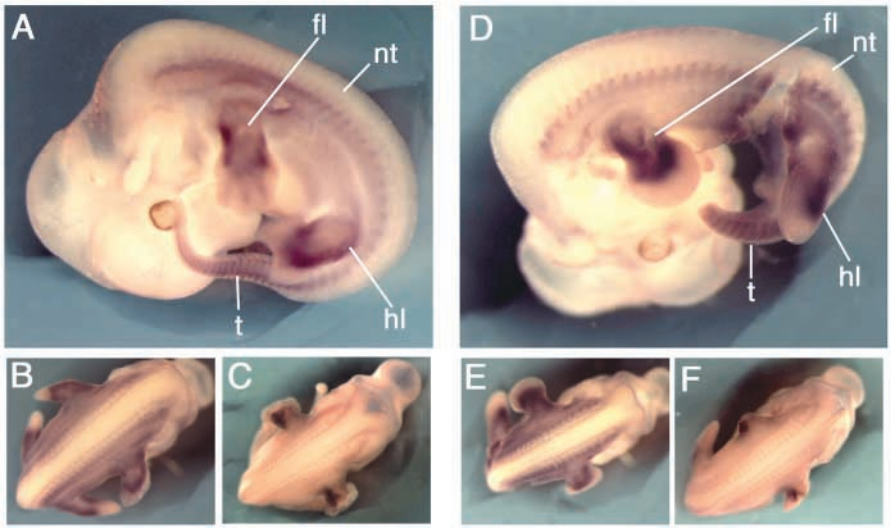

Fig. 4. In situ hybridization for IGF2 and IGFBP4 expression. Wild-type (A,B,C) and PAPPA$^{-/-}$ (D,E,F) E12.5 embryos hybridized with digoxigenin-labeled antisense RNA probes for *IGF2* (A,B,D,E) or *IGFBP4* (C,F). (B,C,E,F) Dorsal views, anterior is to the upper right. Strong expression is seen lateral to the neural tube (nt), most probably in the spinal ganglia, as well as in the forelimbs (fl), hindlimbs (hl) and tail (t).

2). Thus, there was no apparent alteration in expression of IGF ligand, IGFBP4 or type I receptor in PAPPA$^{-/-}$ mice.

Nonetheless, loss of PAPPA and the ability to proteolyze IGFBP4 resulted in impaired IGF-mediated growth responses in MEFs from PAPPA$^{-/-}$ embryos. Impairment in type I IGF receptor tyrosine phosphorylation, the initial event in ligand-mediated receptor activation (LeRoith et al., 1995), is shown in Fig. 5A. MEFs from wild-type and PAPPA$^{-/-}$ embryos were incubated for 48 hours, and then recombinant wild-type or protease-resistant IGFBP4 was added to the medium for another hour of incubation. Cells were washed, lysed and immunoprecipitated with antibody against the type I IGF receptor. Immunoprecipitates were subjected to immunoblotting for tyrosine phosphorylation. Addition of wild-type IGFBP4 had no inhibitory effect on endogenous type I IGF receptor phosphorylation in wild-type MEFs but inhibited receptor phosphorylation in PAPPA$^{-/-}$ MEFs by 70%. Protease-resistant IGFBP4 inhibited type I IGF receptor phosphorylation by 70-80% in both wild-type and PAPPA$^{-/-}$ MEFs. Fig. 5B similarly shows that IGFBP4 had no effect on IGF-stimulated [^{3}H]thymidine incorporation in wild-type MEFs, but inhibited stimulation by 80% in PAPPA$^{-/-}$ MEFs. Stimulation of [^{3}H]thymidine incorporation by IGF analogs with markedly reduced affinity for IGFBP4 (Francis et al., 1993; Oh et al., 1993) was equally effective in wild-type and PAPPA$^{-/-}$ MEFs. IGFBP3, which is not proteolyzed by PAPPA, inhibited IGF-stimulated growth in both wild-type and PAPPA$^{-/-}$ MEFs. These data are consistent with a model of PAPPA increasing local IGF bioavailability and mitogenic effectiveness through cleavage of IGFBP4.

Table 2. IGF stimulation of [^{3}H]thymidine incorporation in mouse embryonic fibroblasts

		% of control	
		Wild type	PAPPA$^{-/-}$
IGF1	2 nM	162±28	139±17
	5 nM	191±18	196±21
	10 nM	224±17	264±13
IGF2	2 nM	154±22	144±36
	5 nM	206±37	200±27
	10 nM	227±41	256±20

Ten separate MEF cultures (five wild type, five PAPPA$^{-/-}$, assayed in triplicate), were treated with the indicated concentrations of IGF1 and IGF2, and [^{3}H]thymidine incorporation determined as described in the Materials and methods. Data (mean±s.e.m., n=5) are expressed as percent of control, i.e. no IGF treatment.

Discussion

The results of this study demonstrate a causal relationship between deletion of the *PAPPA* gene in mice and a growth-deficient phenotype that becomes apparent at least as early as embryonic day 12.5 and persists after birth. Thus, PAPPA, a novel metalloproteinase in the superfamily of metzincins (Boldt et al., 2001), is an important fetal growth regulatory factor in vivo.

In many instances, ablation of one member of a gene family has resulted in compensation by one or more other members. We can argue against complete functional redundancy in this case because specific IGFBP4 protease activity is abolished and PAPPA-null mice have a distinct phenotype, i.e. proportional dwarfism. In addition, there was no apparent compensation in other components of the IGF system in the PAPPA$^{-/-}$ mice, when assessed as follows.

(1) In situ hybridization demonstrated the widespread pattern of *IGF2* and *IGFBP4* mRNA expression during normal embryogenesis, as has been previously reported (Stylianopoulou et al., 1988; Lee et al., 1990; Schuller et al., 1993; Pintar et al., 1998), with no apparent change in spatial pattern or level of mRNA in the PAPPA-deficient embryos. Similarly, there was no significant difference in steady-state levels of *IGF2* and *IGFBP4* mRNA in wild-type and PAPPA$^{-/-}$ embryos as determined by northern analysis.

(2) Serum levels of IGF1 were not different between wild-type and PAPPA-deficient mice.

(3) Fibroblasts from PAPPA-null E13.5 embryos were as responsive as those from wild-type embryos to growth stimulation by IGF1 and IGF2, indicating that capacity for type I IGF receptor signaling was not altered in the absence of *PAPPA* gene expression.

Thus, IGF ligands are expressed, type I IGF receptors are present and operational, and the potential reservoir function of IGFBP4 is preserved in both wild-type and PAPPA$^{-/-}$ embryos. The major difference is the presence or absence of PAPPA and its proteolytic activity.

Although PAPPA-deficient mice were significantly smaller than wild-type littermates, the organ-to-body weight ratios were normal. Thus, the impact of the *PAPPA* mutation could be on

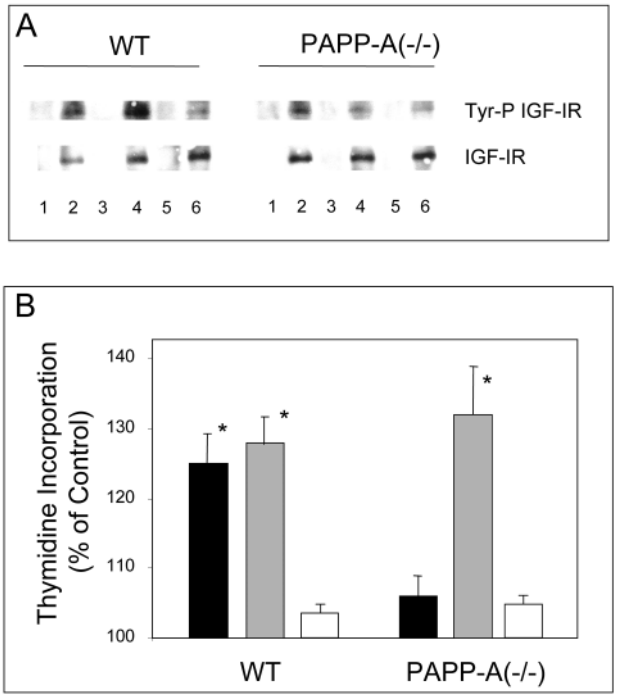

Fig. 5. Effect of PAPPA on IGF-mediated growth responses in mouse embryonic fibroblasts. (A) Fibroblasts from wild-type (WT) and PAPPA$^{-/-}$ embryos were incubated for 48 hours prior to the addition of recombinant wild-type IGFBP4 (lanes 3, 4) or protease-resistant IGFBP4 (lanes 5, 6). Lanes 1, 2 are cells with no IGFBP4 added. After an additional 1 hour of incubation, cells were lysed and immunoprecipitated with either antibody against the type I IGF receptor (even number lanes) or non-specific IgG (odd number lanes), as described in the Materials and methods. Immunoprecipitates were run on SDS-PAGE under reducing conditions and immunoblotted with phosphotyrosine antibody (Tyr-P IGF1R) or type 2GF receptor antibody (IGF1R). (B) Fibroblasts from WT (n=5) and PAPPA$^{-/-}$ embryos (n=8) were incubated for 48 hours prior to the addition of 25 nM IGFBP4 and 5 nM IGF (black bars) or IGF analog (gray bars). Results of experiments using IGF1 and IGF2 [and corresponding analogs, des(1-3)IGF1 and des(1-6)IGF2] were combined as they gave equivalent responses (see Table 2). Addition of 25 nM IGFBP3 and 5 nM IGF is represented by the white bars. [^{3}H]Thymidine incorporation was measured as described in the Materials and methods. Data (mean±s.e.m.) are expressed as percent of control. *, significantly different from control, P<0.01.

control of the timing, rate and/or duration of the growth process early in embryonic development that is important for size control. *S*keletal development experiments indicated striking differences in embryonic size and ossification between wild-type and PAPPA-null embryo littermates as early as E13.5. These findings, together with the findings of developmental delay as early as E12.5 as assessed by in situ hybridization and proportional reduction in organ weights, indicate that the impact of *PAPPA* gene disruption on embryonic growth occurred prior to organogenesis (E10-E14 in the mouse).

The phenotype of the PAPPA-null mouse is strikingly similar to that of the IGF2-null mouse. Thus, mice lacking the ability to express the *IGF2* gene are small at birth and remain 60% of the size of wild-type littermates during post-natal life (DeChiara et al., 1990). They also show a similar delay in skeletal ossification (Liu et al., 1993). The principal period of *IGF2* impact on body size has been suggested to occur during E9-E10 (Burns and Hassan, 2001). In the absence of the *IGF2* gene there are significant decreases in cell proliferation during this period of time that could account for the smaller size of the embryo, detected at ~E12.5. Double mutation studies indicated that IGF2 signals through type I insulin and IGF receptors in early embryogenesis (Baker et al., 1993; Louvi et al., 1997), and a similar growth-deficient phenotype occurs with disruption of *insulin receptor substrate 1*, a key intracellular transducer of type I insulin and IGF receptor signaling (Araki et al., 1994; Tamemoto et al., 1994). The most straightforward explanation for the observed alterations in fetal growth in PAPPA-deficient mice is that PAPPA is necessary for amplification of receptor-mediated IGF2 signaling through specific IGFBP4 proteolysis during a crucial period in embryogenesis. This model is supported by the similar phenotype of PAPPA-, IGF2- and insulin receptor substrate 1-null mice. Interestingly, studies by Pintar et al. indicate that IGFBP4-null mice are 10-15% smaller than wild-type mice (Pintar et al., 1998). This would support the view of IGFBP4 as an important local reservoir of IGFs that can then be released at definitive times and discrete regions through proteolysis. Furthermore, this model is supported by our in vitro studies with fibroblasts from wild-type and PAPPA$^{-/-}$ embryos, demonstrating the importance of PAPPA-induced IGFBP4 proteolysis in regulating endogenous and exogenous IGF bioactivity. Currently, the only identified function of PAPPA is as an IGFBP protease. However, contributions of other IGF system components, as well as possible IGF-independent effects of PAPPA, remain to be determined.

A major conclusion from our genetic data is that PAPPA is clearly involved in optimal fetal growth and development. This growth regulatory mechanism may also underlie the association between PAPPA levels and fetal development recently reported in humans (Smith et al., 2002). Furthermore, PAPPA-deficient mice provide a unique model for testing hypotheses concerning the role of PAPPA in regulating IGF action postnatally, e.g. during bone remodeling and vascular repair (Z. T. Resch, R. D. Simari and C. A. Conover, unpublished).

The authors thank Jeffry Harden, Ralitza Mantcheva and Xiaoshong Wu for all their help with cloning and microinjections, and Christopher Ortiz, Shelley Fohrman and Keira Suttcliffe-Stephenson for their excellent technical assistance. All animal studies were performed under protocols reviewed and approved by Mayo's Institutional Animal Care and Use Committee. This work was supported in part by grants from Novo Nordic Foundation (C.O.) and the Mayo Foundation.

References

Adams, S. O., Nissley, S. P., Handwerger, S. and Rechler, M. M. (1983). Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts. *Nature* **302**, 150-153.

Araki, E., Lipes, M. A., Patti, M.-E., Bruning, J. C., Haag, B., III, Johnson, R. S. and Kahn, C. R. (1994). Pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* **372**, 186-190.

Baker, J., Liu, J.-P., Robertson, E. J. and Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75**, 73-82.

Bayes-Genis, A., Schwartz, R. S., Lewis, D. A., Overgaard, M. T., Christiansen, M., Oxvig, C., Ashai, K., Holmes, D. R., Jr and Conover, C. A. (2001a). Insulin-like growth factor binding protein-4 protease

produced by smooth muscle cells increases in the coronary artery after angioplasty. *Arterioscler. Thromb. Vasc. Biol.* **21**, 335-341.

Bayes-Genis, A., Conover, C. A., Overgaard, M. T., Bailey, K. R., Christiansen, M., Holmes, D. R., Jr, Virmani, R., Oxvig, C. and Schwartz, R. S. (2001b). Pregnancy-associated plasma protein A as a marker of acute coronary syndromes. *N. Engl. J. Med.* **345**, 1022-1029.

Boldt, H. B., Overgaard, M. T., Laursen, L. S., Weyer, K., Sottrup-Jensen, L. and Oxvig, C. (2001). Mutational analysis of the proteolytic domain of pregnancy-associated plasma protein-A (PAPP-A): classification as a metzincin. *Biochem. J.* **358**, 359-367.

Bunn, R. C. and Fowlkes, J. L. (2003). Insulin-like growth factor binding protein proteolysis. *Trends Endocrinol. Metab.* **14**, 176-181.

Burns, J. L. and Hassan, A. B. (2001). Cell survival and proliferation are modified by insulin-like growth factor 2 between days 9 and 10 of mouse gestation. *Development* **128**, 3819-3830.

Byun, D., Mohan, S., Kim, C., Suh, K., Yoo, M., Lee, H., Baylink, D. J. and Qin, X. (2000). Studies on human pregnancy-induced insulin-like growth factor (IGF)-binding protein-4 proteases in serum: determination of IGF-II dependency and localization of cleavage site. *J. Clin. Endocrinol. Metab.* **85**, 373-381.

Byun, D., Mohan, S., Yoo, M., Sexton, C., Baylink, D. J. and Qin, X. (2001). Pregnancy-associated plasma protein-A accounts for the insulin-like growth factor (IGF)-binding protein-4 (IGFBP-4) proteolytic activity in human pregnancy serum and enhances the mitogenic activity of IGF by degrading IGFBP-4 in vitro. *J. Clin. Endocrinol. Metab.* **86**, 847-854.

Chen, B.-K., Lieferman, K. M., Pittelkow, M. P., Overgaard, M. T., Oxvig, C. and Conover, C. A. (2003). Localization and regulation of pregnancy associated plasma protein-A expression in healing human skin. *J. Clin. Endocrinol Metab.* **88**, 4465-4471.

Conover, C. A., Durham, S. K., Zapf, J., Masiarz, F. R. and Kiefer, M. C. (1995). Cleavage analysis of insulin-like growth factor (IGF)-dependent IGF-binding protein-4 proteolysis and expression of protease-resistant IGF-binding protein-4 mutants. *J. Biol. Chem.* **270**, 4395-4400.

Conover, C. A., Faessen, G. F., Ilg, K. E., Chandrasekher, A., Christiansen, M., Overgaard, M. T., Oxvig, C. and Giudice, L. C. (2001). Pregnancy-associated plasma protein-A is the insulin-like growth factor binding protein-4 protease secreted by human ovarian granulosa cells and is a marker of dominant follicle selection and the corpus luteum. *Endocrinology* **142**, 2155-2158.

DeChiara, T. M., Efstratiadis, A. and Robertson, E. J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345**, 78-80.

Firth, S. M. and Baxter, R. C. (2002). Cellular actions of the insulin-like growth factor binding proteins. *Endocr. Rev.* **23**, 824-854.

Francis, G. L., Aplin, S. E., Milner, S. J., McNeil, K. A., Ballard, F. J. and Wallace, J. C. (1993). Insulin-like growth factor (IGF)-II binding to IGF-binding proteins and IGF receptors is modified by deletion of the N-terminal hexapeptide or substitution of arginine for glutamate-6 in IGF-II. *Biochem. J.* **293**, 713-719.

Fuchtbauer, E. M. (1995). Expression of M-twist during postimplantation development of the mouse. *Dev. Dyn.* **204**, 316-322.

Huang, L.-F., Fukai, N., Selby, P. B., Olsen, B. R. and Mundlos, S. (1997). Mouse clavicular development: analysis of wild-type and cleidocranial dysplasia mutant mice. *Dev. Dyn.* **210**, 33-40.

Laursen, L. S., Overgaard, M. T., Søe, R., Boldt, H. B., Sottrup-Jensen, L., Giudice, L. C., Conover, C. A. and Oxvig, C. (2001). Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. *FEBS Lett.* **504**, 36-40.

Lawrence, J. B., Oxvig, C., Overgaard, M. T., Sottrup-Jensen, L., Gleich, G. J., Hays, L. G., Yates, J. R., III and Conover, C. A. (1999). The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. *Proc. Natl. Acad. Sci. USA* **96**, 3149-3153.

Lee, J. E., Pintar, J. and Efstratidais, A. (1990). Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. *Development* **110**, 151-159.

LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, C. T., Jr (1995). Molecular and cellular aspects of the insulin-like growth factor I receptor. *Endocr. Rev.* **16**, 143-163.

Lin, T. M., Halbert, S. P., Kiefer, D. J., Spellacy, W. N. and Gall, S. (1974). Characterization of four human pregnancy-associated plasma proteins. *Am. J. Obstet. Gynecol.* **118**, 223-236.

Liu, J.-P., Baker, J., Perkins, A. S., Robertson, E. J. and Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* **75**, 59-72.

Louvi, A., Accili, D. and Efstratiadis, A. (1997). Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Dev. Biol.* **189**, 33-48.

McClive, P. J. and Sinclair, A. H. (2001). Rapid DNA extraction and PCR-sexing of mouse embryos. *Mol. Reprod. Dev.* **60**, 225-226.

McLeod, M. J. (1980). Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299-301.

Mohan, S., Bautista, C. M., Wergedal, J. and Baylink, D. J. (1989). Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium: a potential local regulator of IGF action. *Proc. Natl. Acad. Sci. USA* **86**, 8338-8342.

Oh, Y., Muller, H. L., Lee, D.-Y., Fielder, P. J. and Rosenfeld, R. G. (1993). Characterization of the affinities of insulin-like growth factor (IGF)-binding proteins 1-4 for IGF-I, IGF-II, IGF-I/insulin hybrid, and IGF-I analogs. *Endocrinology* **132**, 1337-1344.

Ortiz, C., Chen, B.-K., Bale, L., Overgaard, M., Oxvig, C. and Conover, C. A. (2003). Transforming growth factor-β regulation of the insulin-like growth factor binding protein-4 protease system in cultured human osteoblasts. *J. Bone Miner. Res.* **18**, 1066-1072.

Overgaard, M. T., Haaning, J., Boldt, H. B., Olsen, I. M., Laursen, L. S., Christiansen, M., Gleich, G. J., Sottrup-Jensen, L., Conover, C. A. and Oxvig, C. (2000). Expression of recombinant human pregnancy-associated plasma protein-A and identification of the proform of eosinophil major basic protein as its physiological inhibitor. *J. Biol. Chem.* **275**, 31128-31133.

Overgaard, M. T., Sørensen, E. S., Stachowiak, D., Boldt, H. B., Kristensen, L., Sottrup-Jensen, L. and Oxvig, C. (2003). Complex of pregnancy-associated plasma protein-A and the proform of eosinophil major basic protein: disulfide structure and carbohydrate attachment sites. *J. Biol. Chem.* **278**, 2106-2117.

Pintar, J., Schuller, A., Bradshaw, S., Cerro, J. and Grewal, A. (1998). Genetic disruption of IGF binding proteins. In *Molecular Mechanisms to Regulate the Activities of Insulin-Like Growth Factors* (ed. K. Takano, N. Hizuka and S.-I. Takahashi), pp. 65-70. Amsterdam, Holland: Elsevier Science.

Qin, X., Byun, D., Lau, K.-H. W., Baylink, D. J. and Mohan, S. (2000). Evidence that the interaction between insulin-like growth factor (IGF)-II and IGF binding protein (IGFBP)-4 is essential for the action of the IGF-II-dependent IGFBP-4 protease. *Arch. Biochem. Biophys.* **379**, 209-216.

Schuller, A. G. P., van Neck, J. W., Lindenbergh-Kortleve, D. J., Groffen, C., de Jong, I., Zwarthoff, E. C. and Drop, S. L. (1993). Gene expression of the IGF binding proteins during post-implantation embryogenesis of the mouse: comparison with the expression of IGF-I and -II and their receptors in rodent and human. *Adv. Exp. Med. Biol.* **343**, 267-277.

Smith, G. C. S., Stenhouse, E. J., Crossley, J. A., Aitken, D. A., Cameron, A. D. and Connor, J. M. (2002). Early-pregnancy origins of low birth weight. *Nature* **417**, 916.

Soe, R., Overgaard, M. T., Thomsen, A. R., Laursen, L. S., Olsen, I. M., Sottrup-Jensen, L., Haaning, J., Giudice, L. C., Conover, C. A. and Oxvig, C. (2002). Expression of recombinant murine pregnancy-associated plasma protein-A (PAPP-A) and a novel variant (PAPP-Ai) with differential proteolytic activity. *Eur. J. Biochem.* **269**, 2247-2256.

Stewart, C. E. H. and Rotwein, P. (1996). Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. *Physiol. Rev.* **76**, 1005-1026.

Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B. and Bode, W. (1995). The metzincins – topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases. *Protein Sci.* **4**, 823-840.

Stylianopoulou, F., Efstratiadis, A., Herbert, J. and Pintar, J. (1988). Pattern of the insulin-like growth factor II gene expression during rat embryogenesis. *Development* **103**, 497-506.

Tamemoto, H., Kadowaki, T., Tobe, K., Yagi, T., Sakura, H., Hayakawa, T., Terauchi, Y., Ueki, K., Kaburagi, Y., Satoh, S. et al. (1994). Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* **372**, 182-186.

Wetterau, L. A., Moore, M. G., Lee, K.-W., Shim, M. L. and Cohen, P. (1999). Novel aspects of the insulin-like growth factor binding proteins. *Mol. Genet. Metab.* **68**, 161-181.