Expression of pregnancy-associated plasma protein A2 during pregnancy in human and mouse

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Abstract

Pregnancy-associated plasma protein-A and -A2 (PAPPA and PAPPA2) are proteases that cleave IGF binding proteins (IGFBPs) and thereby increase the bioavailability of growth factors. PAPPA has long been recognized as a marker of fetal genetic disorders and adverse pregnancy outcomes. In contrast, although *PAPPA2* is also highly expressed in human placenta, its physiological importance is not clear. To establish whether mice will be a useful model for the study of *PAPPA2*, we compared the patterns of expression of *PAPPA2* in the placentae of mouse and human. We show, for the first time, that *Pappa2* is highly expressed in mouse placenta, as is the case in humans. Specifically, it is expressed at the interface of the maternal and fetal layers of the mouse placenta at all gestational stages studied (10·5–16·5 days post coitum). Similarly, *PAPPA2* is expressed in the syncytiotrophoblast layer of human placental villi and is also detected in some invasive extravillous trophoblasts in the first trimester. These results are consistent with a model whereby PAPPA2 cleaves IGFBPs produced in the maternal decidua to promote feto-placental growth, and indicate that this protein may play analogous roles in human and mouse placenta. PAPPA2 protein is detectable in the circulation of pregnant mice and humans during the first trimester and at term, raising the possibility that PAPPA2 may be a useful biomarker of placental dysfunction. *Pappa2* expression also shows specific localization within the mouse embryo and therefore may play roles in fetal development, independent of its action in the placenta.

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Introduction

The placenta acts as the interface between fetus and mother during gestation (Cross *et al.* 1994). Abnormal placental development leads to serious consequences for both fetal and maternal health, such as intrauterine growth restriction (IUGR; Jackson *et al.* 1995, Salafia *et al.* 1995), and preeclampsia (Redman & Sargent 2005). During the formation of the placenta in primates and rodents, the epithelium of the uterus is eroded by embryonic trophoblasts, which eventually leads to direct contact between maternal blood and fetal trophoblastic villi, facilitating efficient diffusion between maternal and fetal blood (Rossant & Cross 2001). The invasive action of trophoblasts is promoted by growth factors, the bioavailability of which is aided by proteases (Lala & Hamilton 1996, Salamonsen 1999). One of the critical proteases involved in normal placental development is pregnancy-associated plasma protein-A (PAPPA; Sun *et al.* 2002), which is produced by human trophoblasts (Tornehave *et al.* 1984) where it is the major proteolytic enzyme cleaving insulin-like growth factor binding protein-4 (IGFBP4; Boldt & Conover 2007). Degradation of IGFBP4 by PAPPA leads to the release of insulin-like growth factor II (IGF-II; Giudice *et al.* 1998, 2002), which promotes the development of the placenta through its positive influence on trophoblast invasion as well as growth and permeability of this highly specialized organ (Constancia *et al.* 2002). Moreover, PAPPA is secreted into the maternal circulation (Folkersen *et al.* 1981), and can be a useful marker for certain diseases and pregnancy complications. For example, unusually low levels of PAPPA in the first trimester may be indicative of increased risk of fetal genetic disorders such as Down's syndrome (Brambati *et al.* 1993) and Cornelia de Lange syndrome (Aitken *et al.* 1999). Furthermore, low circulating PAPPA levels are also associated with higher risk of low birth weight, IUGR, and preeclampsia (Smith *et al.* 2002, Kwik & Morris 2003, Dugoff *et al.* 2004, Spencer *et al.* 2008), which are characterized by impaired trophoblast invasion. Although *PAPPA* is highly expressed in human placenta (Tornehave *et al.* 1984), this is not the case in the mouse (Qin *et al.* 2002, Soe *et al.* 2002). Nevertheless, *Pappa* plays an important role in fetal development as *Pappa*-deficient mice show delayed and impaired growth (Conover *et al.* 2004).

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Recently, another protease similar to PAPPA has been identified (Farr *et al.* 2000, Overgaard *et al.* 2001, Page *et al.* 2001) and designated as PAPPA2 (earlier names included PAPP-E and Plac3). Like PAPPA, PAPPA2 is abundantly expressed in the human placenta (Farr *et al.* 2000, Page *et al.* 2001), and also cleaves an IGFBP (Overgaard *et al.* 2001). Unlike PAPPA, which proteolyses both IGFBP4 and IGFBP5 (Boldt *et al.* 2004), PAPPA2 cleaves IGFBP5 and may also show lower proteolytic activity against IGFBP3 (Overgaard *et al.* 2001). Although the physiological functions of *PAPPA2* have not been studied, at least four recent studies have found *PAPPA2* to be upregulated in hypertensive disorders of pregnancy including preeclampsia and hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome (Buimer *et al.* 2008, Nishizawa *et al.* 2008, Sitras *et al.* 2009, Winn *et al.* 2009).

Understanding the role of *PAPPA2* in placental growth and development may further enhance our knowledge of disorders such as preeclampsia and IUGR and lead to improvements in diagnosis and therapeutic interventions. Animal models are needed to elucidate the physiological roles of *PAPPA2* in the placenta in health and disease (Rossant & Cross 2001, Sapin *et al.* 2001, Cross 2003). Therefore, the primary goal of the current study was to examine and compare the expression and localization of *PAPPA2* in mouse and human placentae. A secondary goal was to determine its location of expression in mouse embryo.

Materials and Methods

Sample collection

Mice (Charles River Laboratories, Saint-Constant, Quebec, Canada) were housed in the Animal Care Facility at SFU, and all procedures were in accordance with the guidelines of the Canadian Council on Animal Care. Six CD1 female mice were time-mated with CD1 studs, and the day the seminal fluid plug was found was designated as day 0·5 post-coitus (dpc). On 12·5 dpc, females were killed by CO_2 inhalation. Embryos, placentae, stomach, kidneys, and liver were quickly dissected in diethylpyrocarbonate (DEPC)-treated 10×PBS (Nagy *et al.* 2003), and these samples were immediately either frozen at −20 °C for protein work, fixed in 4% paraformaldehyde (PFA) overnight for *in situ* hybridization (placentae and embryos only), or placed in RNAlater (Ambion, Foster City, CA, USA) overnight at 4 °C and then stored at −20 °C prior to quantitative PCR. Nonpregnancy serum samples were collected from live mice from the saphenous vein, and pregnancy serum samples were collected from killed mice by cardiac puncture. Blood samples were centrifuged, and serum was collected and stored at −80 °C until further use.

Serum samples were also obtained from three healthy nonpregnant women, three healthy pregnant women during the first trimester, and three at term. This study was approved by The Ottawa Hospital Research Ethics Board, and informed consent was obtained. Upon collection, samples were centrifuged and the serum frozen at −20 °C for later batch analysis. Early placental tissues were obtained from voluntary terminations at 8–12 weeks.

RNA isolation and quantitative PCR

Quantitative PCR was performed to compare expression levels of *Pappa2* and *Pappa* transcripts. Total RNA was extracted from mouse tissues using QIAshredder homogenizers (Qiagen) and RNeasy spin columns (Qiagen) following the manufacturer's instructions. Each sample was standardized to contain 50 ng/μl total RNA, and a reference sample was prepared by combining aliquots of placental samples; this reference sample was included in every assay to account for variation between assays. The relative expression levels of *Pappa2* and *Pappa* in the tissue samples and reference sample were assessed by quantitative PCR with the MJ Mini Thermal Cycler (Bio-Rad) using primers and probes (Integrated DNA Technologies, Coralville, IA, USA) as described in Table 1. The expression of a 'housekeeping gene', *β-actin* (*Actb*, as given in MGI Database), was also measured. All primers were designed to span introns to avoid detection of genomic DNA. iScript One-Step RT-PCR Kit for Probes (Bio-Rad) was used to reverse-transcribe and amplify the RNA template for 40 cycles, and the cycle at which the signal rose above a fixed threshold (C_t) was determined. The quantitative PCR amplification was performed using 25 μl reaction volumes containing reaction mix (Bio-Rad), Protector RNase Inhibitor (Roche Applied Sciences), 0·5 U iScript RTase, 8·5 μl RNA template (i.e. 425 ng), 0·25 mM each primer, and 175 nM probe. The quantitative PCR program consisted of an initial reverse transcription of 30 min at 50 °C, an initial PCR activation step of 15 min at 95 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C.

Samples were measured in triplicate and the C_t values for *Pappa2* and *Pappa* were normalized to those of *β-actin* using

Table 1 Quantitative PCR primer and probe sequences

Gene	Forward primer	Reverse primer	Probe (contains fluorophore, 6-FAM, on the 5′ end and quencher, BHQ-1, at the 3′ end)
Pappa	CACAATGGACTCTGTGATGCT	TCTCCCTTCTAGGCAAAGGT	TGGTTCCCACCCATCGATGG
Pappa2	GGGACAAGGAAGCTCTCAGT	CAGGGATCATCACAGGATTC	CATGCTTGGCCACACCAACATCATGATCCA
β-Actin	CCTGAAAAGATGACCCAGAT	GGTACGACCAGAGGCATACA	ACCTTCAACACCCCAGCCATGT

the Pfaffl method (2001), which involves calculating the efficiency of the PCR for each gene using serial dilutions of samples. Using this method, expression in a sample is calculated relative to the reference sample measured in the same assay to account for variation between assays.

In order to compare transcript levels between *Pappa2* and *Pappa*, we estimated transcript copy number using a standard curve constructed for each gene. cDNA samples corresponding to the regions amplified during quantitative PCR were generated with a One-Step RT-PCR Kit (Qiagen). The DNA concentration of each cDNA sample was measured using a NanoDrop spectrometer, and cDNA copy number was calculated using cDNA concentration and amplicon length (http://www.uri.edu/research/gsc/resources/cndna.html). cDNA samples were then serially diluted and used as template for quantitative PCR to construct the standard curves.

Western blotting

Mouse tissue samples were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) and incubated on ice for 15 min to allow cell lysis. The homogenates were then centrifuged at 16 000 ***g*** and supernatant collected and stored at −80 °C until further use. Complete Protease Inhibitor Cocktail (Roche Applied Sciences) was added to all samples (including serum samples) to prevent protein degradation.

Samples containing 30 μg total protein were mixed with 5×SDS loading buffer and boiled for 10 min. Samples and pre-stained molecular weight markers (Precision Plus Protein Prestained Standards, Bio-Rad) were loaded onto a 4% stacking gel and run through an 8% polyacrylamide gel under reducing conditions. The gels were then equilibrated in transfer buffer, and proteins were transferred onto nitrocellulose membranes (Bio-Rad) using a semi-dry transfer machine (Bio-Rad). After transfer, the membranes were rinsed, blocked, and then incubated overnight at 4 °C with 1:1000 polyclonal anti-human PAPPA2 antibody (R&D Systems, Minneapolis, MN, USA). According to the manufacturer, this antibody shows <1% cross-reactivity with PAPPA. Membranes were washed, incubated with 1:10 000 fluorescent-labeled secondary antibody (Li-Cor Biosciences, Lincoln, NE, USA) for 45 min at room temperature, rinsed and scanned with an Odyssey infrared imaging system (Li-Cor Biosciences).

For western blotting of human serum, 0·5 μl aliquots of serum samples were resolved using 7·5% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes in duplicate. The two membranes were immunoblotted with anti-human PAPPA and PAPPA2 antibodies (R&D Systems), and the bands were visualized using ECL reagents (Thermo Fisher Scientific, Rockford, IL, USA). To confirm the specificity of the PAPPA2 antibody, a recombinant PAPPA2 peptide (amino acids 243–1396; R&D Systems) was included in the gel as a positive control. Furthermore, an additional membrane was blotted with nonpregnant and pregnant serum samples, which were probed with a secondary antibody (HRP conjugated rabbit anti-goat IgG, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) without incubation with PAPPA2 antibody.

Immunohistochemistry

Cross sections of mouse placenta at different stages of pregnancy were obtained from Zyagen (San Diego, CA, USA). Slides were deparaffinized in xylene washes and rehydrated with graded series of ethanol. Antigen retrieval was performed by heating the slides in a microwave for 15 min in citrate buffer (pH 6·0). Sections were then incubated in PBS with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase. Blocking steps were performed using serum blocking reagent G, avidin blocking reagent, and biotin blocking reagent (R&D Systems), followed by incubation with polyclonal anti-human PAPPA2 or polyclonal anti-mouse IGFBP5 antibody (1:50, R&D Systems) in PBS overnight at 4 °C. After three washes in PBS, samples were incubated with biotinylated secondary antibody (R&D Systems) for 30 min at 37 °C, followed by streptavidin conjugated with HRP, and visualized with 3,3-diaminobenzidine (DAB) substrate (R&D Systems). The slides were then counterstained with hematoxylin. To evaluate the extent of nonspecific immunostaining, primary antibodies were substituted with goat anti-rabbit IgG (1:50; Sigma) as negative controls.

Human placental samples were fixed overnight in 4% PFA–PBS, dehydrated through a graded series of ethanol, and then embedded in paraffin. Adjacent sections (4–5 μm) were deparaffinized in xylene, followed by rehydration in graded series of ethanol concentrations. Sections were washed with PBS and exposed to 15 min of antigen retrieval (400 ml 10 mM citrate buffer, pH 6·0) in the microwave. The inactivation of endogenous peroxidase was performed by the incubation of sections in 3% H_2O_2 with methanol, followed by blocking solution (50 μl) at room temperature. Adjacent sections were then incubated at 4 °C with primary antibody, polyclonal anti-human PAPPA2 antibody (R&D Systems) in 1×PBS. Biotinylated secondary antibodies were applied to samples for 30 min followed by streptavidin–HRP incubation (40 min) at room temperature. Sections were visualized with DAB or 3-amino-9-ethylcarbazole (AEC) under the light microscope. Counterstaining was performed with hematoxylin stain and sectioned slides were mounted with cytoseal 60. To determine the extent of nonspecific immunostaining, primary antibodies were substituted with goat IgG (at the same concentrations) for negative controls.

To further examine whether extravillous trophoblasts express *PAPPA2* at the maternal–fetal interface of human placenta, sections were first stained for cytokeratin 18 (mouse monoclonal antibody, Santa Cruz Biotechnology Inc.), a trophoblast marker, and AEC substrate was used to visualize the immunosignal. After images of typical immunosignals were captured, the sections were destained

with 0·1 M HCl in 70% ethanol to remove rose–red insoluble precipitates. To remove previously applied antibodies, sections were treated with double stain blocker (DAKO Corporation, Mississauga, Ontario, Canada). Sections were further immunostained for vimentin (goat polyclonal antibody, Santa Cruz Biotechnology Inc.), a marker of maternal decidual cells, or PAPPA2 (R&D Systems). The vimentin and PAPPA2 immunosignals in these sections were examined in the same field as the previously captured image that showed cytokeratin immunoreactivity, which allowed the examination of different immunosignals in the same cells of the same sections.

Probe synthesis for in situ *hybridization*

Mouse placental mRNA was reverse transcribed into cDNA using the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas, Burlington, Ontario, Canada), and 600 bp of the *Pappa2* gene was amplified using the following primers: 5′-CAGAGGGAGGACAGAGCAA-3′ and 5′-GTAAAGGTGACAGAATCTCAGG-3′. The 600 bp cDNA was inserted into TA TOPO cloning vector (Invitrogen) and used as template for *in vitro* transcription. A 660 bp fragment of the *Igfbp5* gene was also amplified (forward primer: 5′-ACGAGAAAGCTCTGTCCATGTGTC-3′, reverse primer: 5′-GCTTCATTCCGTACTTGTCCACAC-3′) and cloned into TA TOPO cloning vector. Similarly, a 434 bp fragment of the *Igf-II* (*Igf2* as listed in MGI Database) gene was produced using the following primers: 5′-TTCTCATCTCTTTGGCCTTCGCCT-3′ and 5′-ACGATGACGTTTGGCCTCTCTGAA-3′. Antisense and sense probes were synthesized by either SP6 or T7 RNA polymerase depending on insert orientation. Digoxigenin (DIG)-labeled RNA was subsequently purified by Quick Spin Columns (Roche).

Whole-mount in situ *hybridization*

The brain cavity and heart of mouse embryos were punctured to facilitate exchange of solutions and avoid probe trapping. Embryos were rehydrated by passage through 75, 50, 25% methanol and twice through PBS containing 0·1% Tween-20 (PBST). To permeabilize tissues, embryos were treated with 10 μg/ml proteinase K in PBST for 35 min at room temperature, and rinsed briefly in PBST containing 2 mg/ml glycine. Post-fixation was in 4% PFA with 0·1% glutaraldehyde for 20 min. After one rinse and one wash in PBST, embryos were equilibrated in 1:1 PBST/hybridization mix at room temperature. Hybridization mix was composed of 50% formamide, pH 5 1·3×SSC (Invitrogen), 5 mM EDTA, 50 μg/ml yeast RNA core particle (Sigma), 0·2% Tween-20, 0·5% CHAPS (Sigma), 100 μg/ml heparin (Sigma) and RNAse-free water, adjusted to pH 8 (Correia & Conlon 2001). Embryos were pre-hybridized at 65 °C for at least 1 h and hybridized for 36 h at 65 °C with 1 μg/ml of either antisense or sense (for negative controls) DIG-labeled RNA probes. After hybridization, embryos were rinsed twice, followed by two 30-min washes with pre-warmed hybridization buffer at 65 °C. The solution was then replaced by 0·1 M maleic acid containing 0·1% Tween-20 (MABT) at room temperature. The embryos were incubated in MABT containing 2% Boehringer Blocking Reagent (BBR; Roche) for 1 h at room temperature with gentle shaking, and the solution was replaced with MABT containing 2% BBR and 20% heat-treated goat serum (Sigma) for at least 1 h. The embryos were subsequently incubated with MABT + 2% BBR + 20% sheep serum + 1/5000 dilution of sheep anti-DIG Fab fragment covalently coupled to alkaline phosphatase (Roche) on rotation overnight at 4 °C. The next day, embryos were rinsed three times with MABT, and washed at least five times for 2–4 h each, and incubated overnight with fresh MABT. To visualize probes bound to the embryo, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) was added to the detection buffer consisting of 100 mM NaCl, 100 mM Tris (pH 9·5), and 0·1% Tween-20. The embryos were incubated in the solution until color developed. Subsequently, the embryos were rinsed once and washed at least twice with PBST. Embryos were fixed again in 4% PFA/0·1% glutaraldehyde for 2 h at room temperature. Embryos were then stored in PBST + 0·1% azide at 4 °C (Nagy *et al.* 2003).

Results

Expression of Pappa2 measured by quantitative PCR

Pappa transcript levels were not particularly high in mouse placenta compared to other tissues (Fig. 1A), but *Pappa2* expression in the placenta was much higher than in other tissues (Fig. 1B). For placental samples, the C_t values for *Pappa* ranged from 24 to 28, whereas the C_t values for *Pappa2* ranged from 16 to 19. To estimate the copy number of each gene transcript in placenta samples, C_t values were converted into

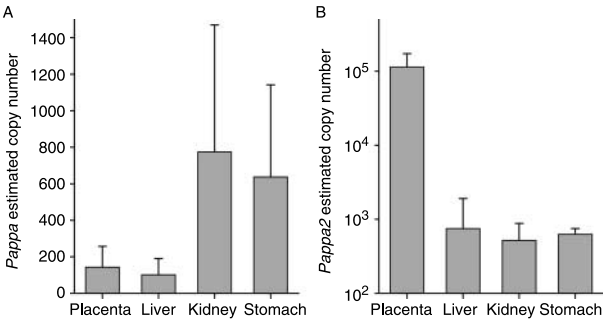

Figure 1 Relative mRNA expression levels of (A) *Pappa* and (B) *Pappa2* in mouse placenta ($n=6$), liver ($n=3$), kidney ($n=3$), and stomach ($n=3$). Values are expressed as estimates of cDNA copy number (calculated using standard curves described in text), and therefore comparison between genes assumes that reverse transcription reactions were equally efficient for *Pappa* and *Pappa2*. Each column represents the mean ± S.D.

copy number using standard curves constructed by plotting the log-transformed value of initial cDNA copy number (calculated from cDNA concentration) against its C_t value. The r^2 values were 0·9951 for *Pappa* and 0·9933 for *Pappa2*, showing a strong correlation between C_t values and copy number. The copy number of *Pappa2* in the placenta was estimated to be nearly 1000-fold higher than that of *Pappa* (*Pappa*: 141 ± 149 copies; *Pappa2*: $1{\cdot}3 \times 10^5 \pm 0{\cdot}7 \times 10^5$ copies). Because this analysis compares cDNA copy number, rather than mRNA copy number, this comparison assumes that the reverse transcription reaction was equally efficient for both *Pappa* and *Pappa2*.

Western blotting

Western blot analysis (Fig. 2) showed that PAPPA2 protein was strongly expressed in the murine placenta and to a lesser extent in the embryo, and that it was also detectable in murine pregnancy serum. It was not possible to use pseudopregnant females to test whether the placenta was the source of serum PAPPA2 in pregnant females because pseudopregnancy does not usually last until 12·5 dpc in mice (Jasper *et al.* 2000, Miller *et al.* 2004). PAPPA2 protein was not detectable in nonpregnant mouse serum, liver, kidney, and stomach (Fig. 2). The pattern of expression was similar in all tested mice.

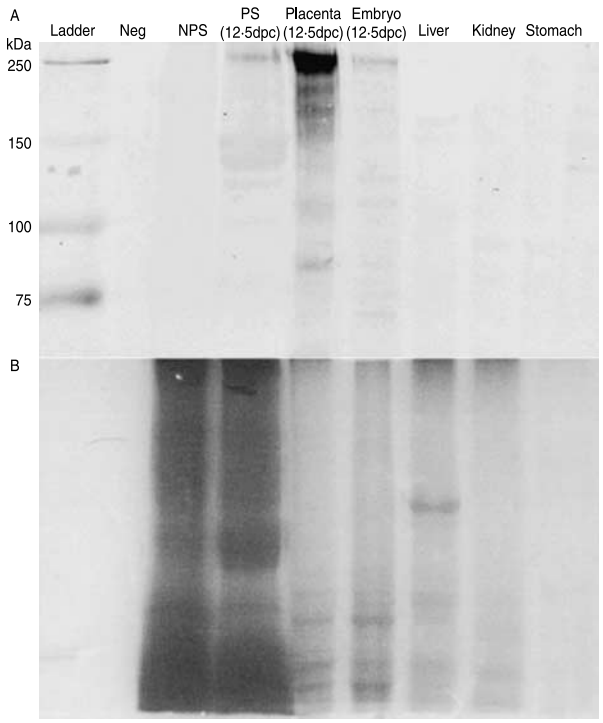

Figure 2 (A) Western blot of PAPPA2 in various murine tissues. The blot shown is representative of blots obtained from six different mice (neg:, negative control (water); NPS, nonpregnancy serum; PS, pregnancy serum; dpc, day post coitum). (B) EZblue-stained SDS-PAGE gel loaded with the same protein samples as used for immunodetection.

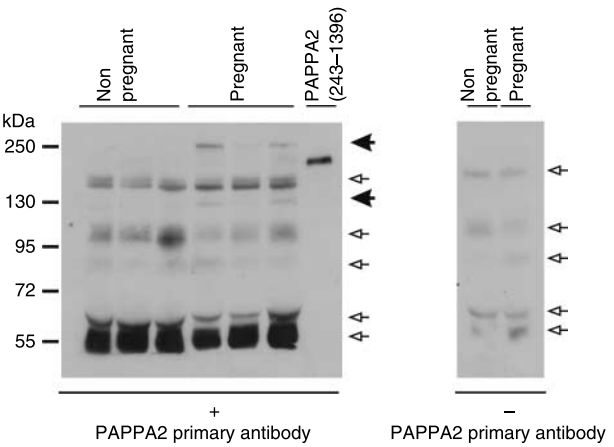

Figure 3 Western blot of PAPPA2 in serum from pregnant women at term and nonpregnant women. Left panel: two protein bands of ~250 and 130 kDa (indicated with solid arrows) are detected in pregnant serum samples but not in nonpregnant samples, representing full-length PAPPA2 and its processed fragment respectively. Right panel: the other bands (indicated with open arrows) are also present in the western blot without anti-PAPPA2 antibody incubation, suggesting nonspecific binding. The positive control (last lane of left panel) consists of a recombinant fragment of PAPPA2 (amino acids 243–1396; R&D Systems) and therefore is smaller than the full-length PAPPA2 in serum samples.

In humans, PAPPA2 was detected in human serum during pregnancy at term (Fig. 3) and at lower levels during the first trimester (Fig. 4), but not in serum from nonpregnant women (Figs 3 and 4). The estimated size of PAPPA2 was ~250 kDa in both mouse and human (Figs 2 and 3), as found by Nishizawa *et al.* (2008). In humans there was also a band of ~130 kDa, which is close to the expected size of a splice variant of PAPPA2 (Page *et al.* 2001).

Localization of PAPPA2 in the placenta

The spatial and temporal expression pattern of PAPPA2 protein in the mouse placenta from gestational stages 10·5–16·5 dpc was determined by immunohistochemistry. The mouse placenta is composed of three main layers: a layer of decidual cells of maternal origin, the labyrinth of fetal origin, and the intermediate junctional zone. Throughout the stages examined, PAPPA2-positive signals were detected primarily in the junctional zone (Fig. 5). Because we observed PAPPA2 expression in the junctional zone but not the decidua, we did not examine pseudopregnant controls, in which the junctional zone would not be present. At 11·5 dpc, IGFBP5 was expressed in the decidual part of the placenta, adjacent to PAPPA2 expression (Fig. 5). In human placenta, PAPPA2 was clearly detectable in the syncytiotrophoblast layer of placental villi in the first trimester (see Supplementary Figure 1 in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/cgi/content/full/JOE-09-0136/DC1) and in invasive extravillous trophoblasts at the maternal–fetal interface (Fig. 6).

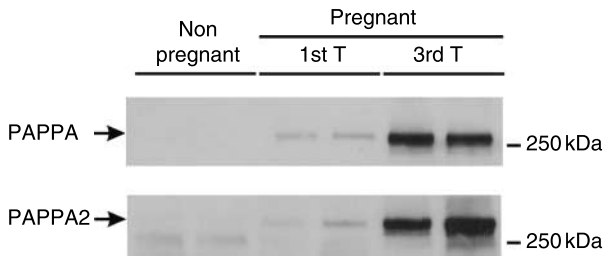

Figure 4 Western blot of PAPPA and PAPPA2 in maternal circulation during the first and third trimester (T).

Whole-mount in situ *hybridization of mouse embryo*

At embryonic day 12·5, *Pappa2* transcripts were found to be present in the nasal region, forebrain, dorsal side, and the sides of the tail (Fig. 7A and B). To compare the expression of *Pappa2* with that of its substrate, *in situ* hybridization was also carried out with *Igfbp5* RNA probes. *Igfbp5* transcripts were expressed throughout the body except in the brain region, developing whisker barrels and feet (data not shown), as found by Allan *et al.* (2000). *Igfbp5* expression in the tail occurred laterally and along the midline (Fig. 7C). There was some co-expression of *Pappa2* and *Igfbp5* in the tail and nasal region. *Igf-II* was also found to be expressed in the tail, as well as the limbs (Fig. 7D).

Discussion

In the present work, we show for the first time that *Pappa2*, but not *Pappa*, is highly expressed in the mouse placenta. *Pappa2* is expressed at much higher levels in the murine placenta than in other tissues analyzed, such as the kidney, stomach, and liver. Previously, *Pappa2* expression was found to be higher in stomach than in a variety of other adult tissues, including brain, kidney, heart, lung, testis, pancreas, and prostate gland (Christians *et al.* 2006). In humans, *PAPPA2* has also been expressed much more strongly in placenta than in other adult tissues (Farr *et al.* 2000, Page *et al.* 2001). In contrast, while *PAPPA* is highly expressed in human placenta (Sun *et al.* 2002), *Pappa* expression in the murine placenta is not particularly high compared to other tissues (Qin *et al.* 2002, Soe *et al.* 2002, this study). We quantitatively compared the transcript abundance of *Pappa* and *Pappa2* in mouse placenta and found that *Pappa2* levels were nearly 1000-fold higher than those of *Pappa*, as estimated by cDNA levels.

To identify specific sites of PAPPA2 expression, immunohistochemistry was performed in both mouse and human placentae. In the mouse, PAPPA2 is primarily expressed in the junctional zone, located between the maternal decidua and the fetal labyrinth zone. The junctional zone is mainly

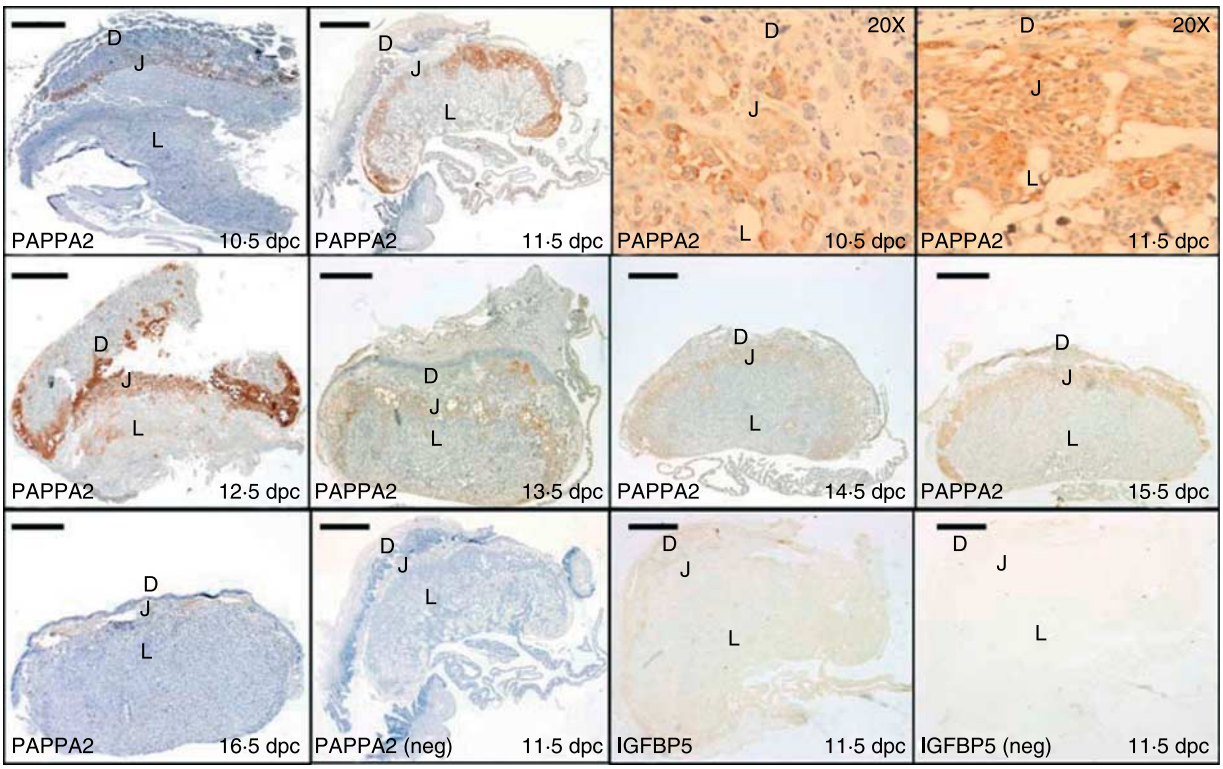

Figure 5 Expression of PAPPA2 is strongest in the junctional zone in the mouse placenta. IGFBP5 protein is expressed in the decidual part in the mouse placenta at 11·5 dpc. No staining was observed when nonimmune goat IgG was used for immunohistochemical detection in mouse placenta (neg). D, decidua; J, junctional zone; L, labyrinth; and dpc, day post coitum. Note that the sections showing IGFBP5 expression were not counterstained with hematoxylin due to low signal level. The scale bar represents 1 mm.

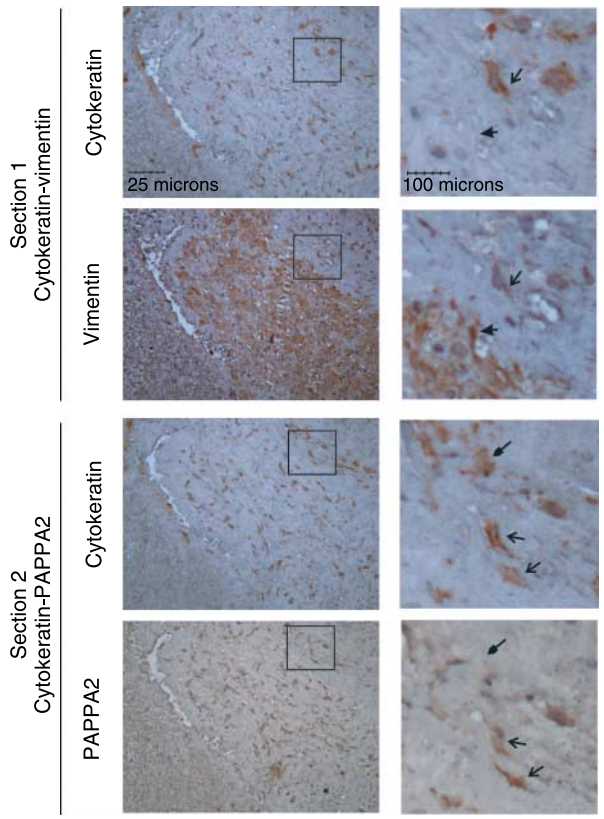

Figure 6 PAPPA2 expression in invasive extravillous trophoblasts at 12 weeks. Sections were initially immunostained for cytokeratin 18, a marker of trophoblasts, followed by destaining and restaining for vimentin, a marker of maternal decidual cells, or PAPPA2. As shown in section 1, cytokeratin-positive cells are vimentin-negative (indicated by line arrowheads), whereas cells displaying vimentin immunoreactivity did not show cytokeratin immunoactivity (indicated by solid arrowheads), confirming the specificity of cytokeratin as a trophoblast marker and no interference of initial cytokeratin staining on subsequent immunostaining. Most cytokeratin-positive cells displayed PAPPA2 immunosignals (indicated by line arrowheads), although some trophoblasts (indicated by solid arrowheads) lacked PAPPA2 immunoreactivity.

composed of two cell types: the spongiotrophoblasts and glycogen trophoblast cells (Coan *et al.* 2005). Glycogen trophoblast cells have been proposed to be a specialized subtype of spongiotrophoblast, involved in the invasion of the decidua by fetal tissue (Adamson *et al.* 2002). The substrate of PAPPA2, IGFBP5, is expressed in some maternal components of the mouse placenta, including endothelium of maternal blood vessels (Carter *et al.* 2006, this study). Given that PAPPA2 contributes to the proteolysis of IGFBP5 (Overgaard *et al.* 2001), its expression in the junctional zone likely leads to the breakdown of IGFBP5 in the neighboring decidua, freeing IGF-II. Similarly, in the human placenta, PAPPA2 is expressed in the syncytiotrophoblast layer of placental villi in the first trimester and may contribute to the release of IGF-II to promote feto-placental growth. In general, our results with human placenta confirm previous findings (Nishizawa *et al.* 2008, Winn *et al.* 2009). However, using cell-specific markers to distinguish between trophoblasts and maternal decidual cells, we also observed some PAPPA2 immunoreactivity in invasive extravillous trophoblasts in the first trimester (at 12 weeks).

PAPPA2 is detectable in the circulation of pregnant women during the first trimester (Nishizawa *et al.* 2008; this study) raising the possibility that PAPPA2 may be a useful biomarker of placental dysfunction, as is the case with PAPPA. At least four recent studies have found *PAPPA2* to be upregulated in hypertensive disorders of pregnancy including preeclampsia and HELLP syndrome (Buimer *et al.* 2008, Nishizawa *et al.* 2008, Sitras *et al.* 2009, Winn *et al.* 2009). It is curious that, although PAPPA2 and PAPPA are proteases that cleave IGFBPs, upregulation of *PAPPA2* is associated with disease, whereas abnormally low maternal serum levels of PAPPA are associated with a variety of pregnancy complications (Smith *et al.* 2002, Kwik & Morris 2003, Dugoff *et al.* 2004, Spencer *et al.* 2008). The evaluation of PAPPA2 as a biomarker will require a better understanding of its physiological roles in the placenta, which will benefit from animal models (Sapin *et al.* 2001, Cross 2003). The parallels in the location of *PAPPA2* expression between mouse and human found in this study indicate that the mouse will provide a suitable model.

Pappa2 is also expressed in mouse embryo. Using whole-mount *in situ* hybridization, we found that *Pappa2* is expressed in the tail and dorsal side of the embryo, forebrain, and the nasal region. While *Pappa* is present throughout the embryonic tail (Conover *et al.* 2004), *Pappa2* transcripts are laterally located in the tail and the dorsal side of the embryo. The substrate of PAPPA2, IGFBP5, has been found to be expressed in various embryonic tissue types, including the developing tail (Allan *et al.* 2000), as confirmed in this study. The co-localization of PAPPA2 and IGFBP5 in certain embryonic tissues suggests that PAPPA2 may play a role in

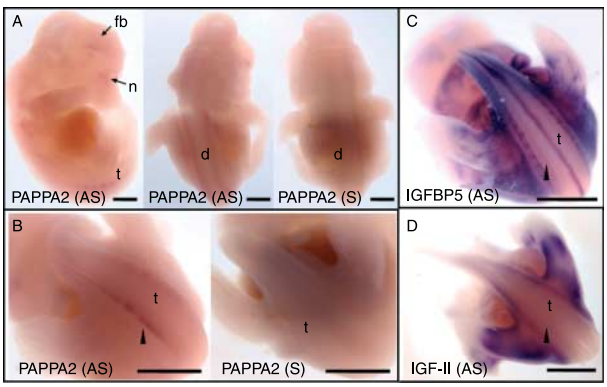

Figure 7 *Pappa2*, *Igfbp5*, and *Igf-II* expression in mouse embryos at e12·5. Low levels of *Pappa2* transcripts are present in the nasal region (n), forebrain (fb), dorsal side (d), and tail (t) (A and B). The same embryos are shown in A and B. The expression of *Pappa2* in the tail laterally co-localizes with that of *Igfbp5* (C), which in turn also colocalizes with *Igf-II* expression (D) (indicated by arrowheads). AS, antisense probes; S, sense probes. The scale bar represents 1 mm.

modulating IGFBP5 bioavailability in the mouse fetus, potentially affecting prenatal growth. Previous work with mice has suggested that *Pappa2* may play a role in postnatal growth; *Pappa2* was identified as a candidate gene for a quantitative trait locus (QTL) affecting tail length, skeletal growth, and levels of circulating IGFBP5 in mice (Christians *et al*. 2006, Christians & Senger 2007). The localization of *Pappa2* expression in the embryonic tail (this study) is consistent with the hypothesis that *Pappa2* is the gene responsible for a QTL affecting tail length.

This study demonstrates that *Pappa2* is highly expressed at the feto-maternal interface of mouse placenta, confirming that the mouse will likely be a useful model for understanding the role of *PAPPA2* in placental physiology. Previous observations show that PAPPA2 is found at higher levels in pregnancies complicated by preeclampsia or HELLP syndrome, whereas PAPPA is found at lower levels in various pregnancy complications which indicate that these two proteins may play quite different physiological roles despite having similar biochemical actions. The development of PAPPA2 as an additional marker may therefore offer increased diagnostic power for identifying placental dysfunction and complications such as preeclampsia and IUGR, which have lasting health consequences for the mother and child.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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