

# Eosinophils are cellular targets of the novel uteroplacental heparin-binding cytokine decidual/trophoblast prolactin-related protein

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## Abstract

The uterus and placenta of the mouse and rat produce a member of the prolactin (PRL) family referred to as decidual/trophoblast PRL-related protein (d/tPRP). This cytokine/hormone has been hypothesized to regulate decidual cell activities needed for the establishment and maintenance of gestation. An alkaline phosphatase (AP)-tagging strategy was used to identify d/tPRP target cells. AP-d/tPRP bound to virtually all cells and tissues to which it was exposed, consistent with our earlier evidence that d/tPRP binds to heparin-containing molecules. Moreover, we found that co-incubation with heparin or pretreatment with heparitinase greatly decreased the binding of AP-d/tPRP to tissue sections. In addition, we observed that the AP-d/tPRP probe bound to the surface of Chinese hamster ovary (CHO) cells but not to heparan sulfate-deficient CHO-pgsD-677 cells. Potential unique non-heparin d/tPRP binding sites within mouse and rat uteroplacental tissues were identified by consecutively

incubating sections with AP-d/tPRP followed by heparin. This strategy led to the identification of d/tPRP target cells associated with the uterus and the labyrinth zone of the chorioallantoic placenta. Within the uterus, d/tPRP specifically bound to eosinophils. d/tPRP-binding and eosinophil peroxidase activity were co-localized and showed similar patterns of distribution during the estrous cycle, pregnancy, and following hormonal manipulation. d/tPRP interactions with eosinophils were further demonstrated in the lung and intestine, with eosinophils isolated from the peritoneum, and in mice with generalized tissue eosinophilia. Collectively, these findings suggest that intercellular d/tPRP targeting is mediated through associations with heparin-containing molecules which help direct d/tPRP to specific interactions with eosinophils within the uterus and with the labyrinthine compartment of the chorioallantoic placenta.

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## Introduction

The establishment of pregnancy requires numerous concomitant maternal uterine changes, including those related to the vasculature and immune cells. Hemochorial placentation occurs in both primates and rodents, resulting in the establishment of a close connection between maternal and fetal tissues (Enders & Welsh 1993). This close connection facilitates the exchange of nutrients and wastes at the expense of an increased risk of maternal immune system recognition of the genetically disparate embryo. Decidual and trophoblast cells, and their secretory products, likely provide the signaling system that coordinates the activities of the maternal uterine compartment.

Decidual cells arise from uterine stroma, forming intimate relationships with placental structures that facilitate the development of the embryo (DeFeo 1967, Bell 1983, Parr & Parr 1989). Among the important functions of decidual cells are their hormone/cytokine producing capabilities. In the mouse and rat, decidual cell production of a hormone related to pituitary prolactin (PRL) is of particular interest. This hormone, referred to as decidual/trophoblast PRL-related protein (d/tPRP), has been implicated in directing early changes in the uteroplacental compartment required for the establishment of pregnancy (Roby *et al.* 1993, Rasmussen *et al.* 1996). d/tPRP was originally isolated during a search for a putative decidual luteotropin (Roby *et al.* 1993). Although d/tPRP exhibits significant structural similarities to PRL,

it does not interact with PRL receptors and thus its putative luteotropic actions are suspect (Rasmussen *et al.* 1996). d/tPRP is predominantly expressed by decidual cells during the first half of pregnancy and subsequently by spongiotrophoblast and trophoblast giant cells of the chorioallantoic placenta during the second half of pregnancy (Roby *et al.* 1993, Gu *et al.* 1994, Lin *et al.* 1997, Orwig *et al.* 1997a,b; Rasmussen *et al.* 1997). Once secreted from its cellular source, d/tPRP readily associates with heparin-containing molecules and accumulates in the extracellular matrix (Rasmussen *et al.* 1996). Thus, d/tPRP is strategically positioned to interact with various populations of cells traversing the uteroplacental compartment (i.e. endothelial, immune and trophoblast cells).

The purpose of this investigation was to specifically identify cellular targets for d/tPRP. We have utilized an alkaline phosphatase (AP) tagging strategy that has been effectively used for identifying targets for other members of the PRL family (Müller *et al.* 1998, 1999). We show that AP-d/tPRP binds specifically to cells within the uterus and in the developing chorioallantoic placenta, including infiltrating eosinophils.

Eosinophils contribute actively to inflammatory responses through their secretion of a variety of chemical mediators (Gleich *et al.* 1993, Venge 1993, Weller 1993, Hirai *et al.* 1997, Rothenberg 1998, Giembycz & Lindsay 1999). These cells are actively recruited to sites of allergic responses (Kay *et al.* 1997), tumors (Tepper *et al.* 1992) and parasite infestation (Pearlman 1997). Eosinophil dynamics are precisely controlled within the uterus by the hormonal milieu, and exhibit dramatic fluctuations during the reproductive cycle (Rytomaa 1960, Bassett 1962, Ross & Klebanoff 1966, McMaster *et al.* 1992, Jeziorska *et al.* 1995). For example, estrogen stimulates the infiltration of eosinophils into the uterus, whereas uterine exposure to progesterone is associated with eosinophil exit and death (Bjersing & Borglin 1964, King *et al.* 1981, Tchernitchin 1983, Lee *et al.* 1989, Howe *et al.* 1990). The present study suggests that since d/tPRP specifically interacts with eosinophils, this cytokine is a candidate mediator of the anti-inflammatory actions of progesterone during gestation.

## Materials and Methods

### Reagents

Supplies for polyacrylamide gel electrophoresis were purchased from Bio-Rad (Hercules, CA, USA). All restriction enzymes, polymerases, and DNA ligase were purchased from New England Biolabs (Beverly, MA, USA). Transformation competent DH $\alpha$ 5 bacterial cells, *Pfu* polymerase were acquired from Stratagene (La Jolla, CA, USA). DNA extraction kits were purchased from Qiagen (Chatsworth, CA, USA). FuGene 6 transfection

reagent was obtained from Boehringer Mannheim (Indianapolis, IN, USA). T7 DNA sequencing kits were acquired from United States Biochemical (Cleveland, OH, USA). The pCMV/SEAP vector was acquired from Tropix Inc. (Bedford, MA, USA). The pECE/long PRL receptor expression vector was a generous gift from Dr Paul Kelly (INSERM, Paris, France). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Rat anti-mouse macrophage antibody, F4/80, was obtained from Dr Stephen Russell of the University of Kansas Medical Center (Kansas City, KS, USA). Antibodies to rat major basic protein were obtained from Dr M J Dembele-Duchesne (INSERM, Montpellier, France). Mouse anti-rat macrophage antibodies, ED1 and ED2, were obtained from Harlan Bioproducts (Indianapolis, IN, USA). Avidin-biotin-immunoperoxidase kits were purchased from Zymed Laboratories (South San Francisco, CA, USA). Reagents for the detection of immune complexes by enhanced chemiluminescence were acquired from Amersham Corp. (Arlington Heights, IL, USA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma (St Louis, MO, USA).

### Animals and tissue preparation

Holtzman rats and CD-1 mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). The IL-5 transgenic mouse line (NJ-1638) was established and propagated as previously described (Lee *et al.* 1997b). Timed pregnancies, pseudopregnancies and tissue dissections were performed as previously described (Soares *et al.* 1983, Soares 1987, Roby *et al.* 1993, Orwig *et al.* 1997b). The presence of a copulatory plug was designated day 1 of pregnancy. Decidual responses were induced in pseudopregnant mice by injection of 50  $\mu$ l sesame oil per uterine horn on day 4 of pseudopregnancy (Orwig *et al.* 1997b). In some experiments, prepubertal rats (day 20 postnatal) were subcutaneously injected daily for 3 days with estradiol valerate (100  $\mu$ g/100  $\mu$ l; Bristol-Myers Squibb, Princeton, NJ, USA) or vehicle (sesame oil). Twenty-four hours following the last injection, rats were killed, uterine tissues dissected, and frozen for subsequent analysis. In all experiments, at least three different tissue samples were examined from a minimum of three different animals. Protocols for the care and use of animals were approved by the University of Kansas Animal Care and Use Committee.

### Cell culture

Human embryonic kidney (HEK) 293 cells were used as a host for the expression of the AP-d/tPRP fusion protein. HEK 293 cells were routinely maintained in minimum essential medium (MEM) supplemented with 20 mM

HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). CHO cells, heparan sulfate-deficient CHO-pgsD-677 cells (Lidholt *et al.* 1992), and d/tPRP-expressing CHO cells (Rasmussen *et al.* 1996) were maintained in Dulbecco's MEM/MCDB 302 culture medium containing 100 units/ml penicillin, 100 µg/ml streptomycin and 10% FBS. All cells were cultured in an atmosphere of 5% CO<sub>2</sub>-95% air at 37 °C in a humidified incubator.

#### *Generation and characterization of the AP-d/tPRP fusion protein*

A fusion protein consisting of a modified human placental AP (PLAP) and rat d/tPRP was generated and used to monitor d/tPRP target cell interactions. Procedures for generating the fusion protein were similar to those previously reported for the generation of an AP-placental lactogen-I (AP-PL-I) fusion protein (Müller *et al.* 1998). A vector containing ampicillin and neomycin resistance genes and a secreted version of PLAP (SEAP; Berger *et al.* 1988) situated downstream of a CMV promoter (pCMV/SEAP) was commercially obtained (Tropix). A nucleotide region representing the mature rat d/tPRP protein was then amplified and ligated into the pCMV/SEAP vector. Ligation with the d/tPRP insert resulted in a CMV promoter driven vector containing the ligated cDNAs encoding a SEAP-d/tPRP fusion protein (AP-d/tPRP). DNA sequencing of the insert was performed to verify the accuracy of the PCR amplification. After linearization, the AP-d/tPRP construct was electroporated into HEK 293 cells. Following a 2-week selection with 500 µg/ml G418, single clones were isolated by limiting dilution and screened for secreted heat stable AP activity. An unmodified pCMV/SEAP vector (AP) was similarly transfected, selected and served as a negative control. After the cells reached confluency, the culture medium was changed to serum-free MEM+HEPES, further conditioned for 72 h, collected and clarified by centrifugation, and stored at -20 °C until used. AP activity was measured from conditioned medium via a colorimetric assay (Müller *et al.* 1998). Western blot analysis for d/tPRP was performed as previously described (Rasmussen *et al.* 1996). AP-d/tPRP preparations were isolated from conditioned medium using immunoprecipitation with monoclonal antibodies to AP conjugated to agarose. PRL-like biological activities were assessed through the use of the rat Nb2 lymphoma cell proliferation assay (Tanaka *et al.* 1980, Deb *et al.* 1993) and through incubation with CHO cells transiently transfected with the long form of the rat PRL receptor (pECE/long, Ali *et al.* 1992).

#### *Analysis of AP-d/tPRP binding to tissues and cells*

Tissues were frozen in dry ice-cooled heptane and stored at -70 °C until tissue sections (8-10 µm) were prepared

with the aid of a cryostat. Sections were mounted onto glass slides, washed in a modified Hank's balanced salt solution (HBHA; containing 20 mM HEPES, 0.5 mg/ml BSA and 0.1% NaN<sub>3</sub>) and incubated with AP, AP-d/tPRP, or AP-d/tPRP+excess recombinant rat d/tPRP for 75 min. Recombinant rat d/tPRP was generated in CHO cells and purified as previously described (Rasmussen *et al.* 1996). Following incubation, the sections were washed with HBHA supplemented with 0.1% Tween 20 and fixed for 2 min in 20 mM HEPES buffer containing acetone (60%) and formaldehyde (3%). The fixed sections were washed, heated at 65 °C for 30 min to inactivate endogenous tissue AP activity, and then processed for detection of the heat stable AP activity associated with the fusion proteins (Müller *et al.* 1998). For *in situ* AP detection 5-bromo-4-chloroindoxyl phosphate substrate and Nitroblue Tetrazolium were used. In experiments designed to co-localize AP-d/tPRP-binding and F4/80-immunoreactive cells, AP-fusion protein binding was assessed with a mouse monoclonal antibody to human PLAP (clone 8B6) and FITC-labeled goat anti-mouse IgG antibodies. Distinction of AP-d/tPRP binding to heparin-containing molecules from other potential interactions was achieved by treating tissue sections with heparin (250 µg/ml to 10 mg/ml) for 5 min following the HBHA and 0.1% Tween 20 washes. AP-d/tPRP binding to tissue sections was also evaluated following preincubation with heparitinase (5 units/ml) at 37 °C for 1 h. The specificity of binding was further assessed by incubation with the AP control or by the addition of recombinant d/tPRP. AP-d/tPRP binding was also evaluated in various leukocytic cell populations (including eosinophils and macrophages) isolated from the rat peritoneum. Finally, AP-d/tPRP binding was assessed in CHO cells transiently transfected with an expression vector containing the long form of the rat PRL receptor (pECE/long, Ali *et al.* 1992) using FusGene 6 according to the manufacturer's instructions. The AP-PL-I fusion protein (Müller *et al.* 1998) was used as a positive control for binding to the rat PRL receptor.

#### *Isolation of rat peritoneal cells*

Peritoneal serosal cells from rats were collected by lavage of the peritoneal cavity with MEM containing 15 mM HEPES, 10% FBS and heparin (50 µg/ml; HMEM) as previously described (Dileepan *et al.* 1993). After gentle massage for 1 min, the peritoneal fluid was aspirated and the cells washed twice with HMEM. Macrophages and eosinophil fractions were isolated based on the procedure of MacKenzie *et al.* (1981). Briefly, peritoneal cells were cultured for 2 h in 75 cm<sup>2</sup> plastic tissue culture flasks. Of the adherent cells >95% were macrophages. Non-adherent cells were collected, washed and resuspended in HMEM at a concentration of 10<sup>7</sup> cells/ml. Cells were further fractionated over a metrizamide gradient.

Metrizamide was made up to 18.5% and 22.5% (w/v) solution in HBSS (Hank's balanced salt solution). Two milliliters of the 22.5% metrizamide solution were overlaid with 2 ml of the 18.5% solution in a 15 ml centrifuge tube. One milliliter of the  $1 \times 10^7$  cells/ml suspension of non-adherent cells was added and the tube centrifuged at room temperature for 15 min at 400 g. Eosinophils were collected at the 18.5%–22.5% metrizamide interface at a purity of 85–90%.

#### Immunocytochemical and histochemical analyses

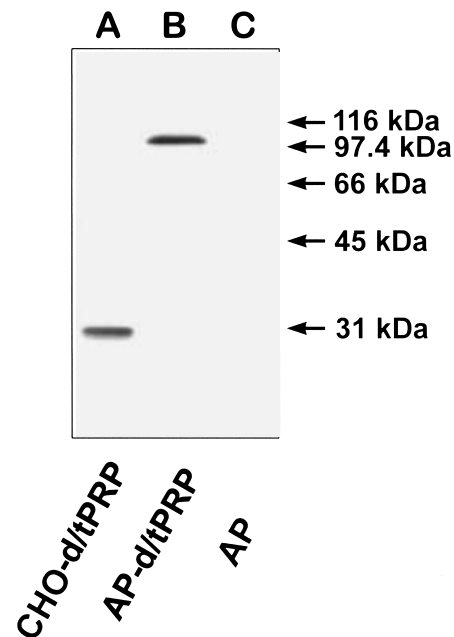
d/tPRP protein expression was monitored by immunocytochemistry with antibodies to rat d/tPRP (Rasmussen *et al.* 1996). Immunocytochemical identification of rat and mouse eosinophils utilized rabbit polyclonal antibodies to mouse and rat major basic protein respectively (Dembele-Duchesne *et al.* 1991, Lee *et al.* 1997a). Eosinophils were histochemically identified by the presence of cyanide-resistant (8 mM NaCN) peroxidase activity as previously described (Horton *et al.* 1996). For identification of cells of the mouse macrophages/eosinophil lineage a rat monoclonal antibody referred to as F4/80 was used (Austyn & Gordon 1981, McGarry & Stewart 1991), whereas rat macrophages were identified with mouse monoclonal antibodies referred to as ED1 and ED2 (Dijkstra *et al.* 1985). Immunocytochemistry was performed with streptavidin–biotin–immunoperoxidase kits using 3-amino-9-ethyl carbazole as a substrate. Control sections were incubated with non-immune serum or non-immune IgG.

## Results

#### Generation and characterization of the AP–d/tPRP fusion protein

We generated and characterized an AP–d/tPRP fusion protein by in-frame insertion of the cDNA sequence of mature rat d/tPRP downstream from the SEAP coding sequence within the pCMV/SEAP vector (Tropix Inc.). The AP–d/tPRP construct and an unmodified AP control vector were transfected via electroporation into HEK 293 cells. The presence of AP activity in conditioned medium from transfected and non-transfected cells was evaluated using a colorimetric assay. Western blot analysis showed that d/tPRP antibodies recognized an AP–d/tPRP protein species approximating 110 kDa and native d/tPRP protein species of 29 kDa (Fig. 1). The 110 kDa  $M_r$  of the AP–d/tPRP fusion protein was consistent with the predicted  $M_r$  of its AP and d/tPRP components. The AP-only control preparation was not recognized by the d/tPRP antibodies.

The AP–d/tPRP fusion protein was evaluated for its ability to interact with heparin-containing cells and the

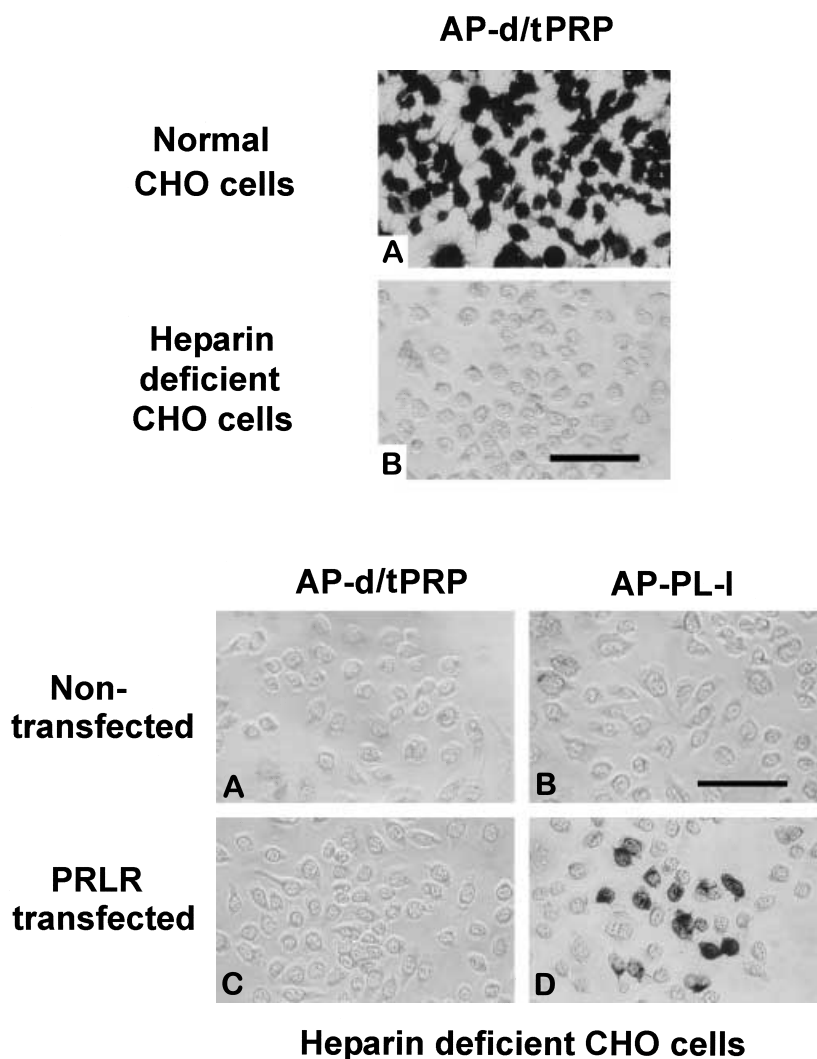


**Figure 1** Characterization of the AP–d/tPRP expression vector. A fusion protein consisting of a modified human placental alkaline phosphatase (AP) and rat d/tPRP was generated and used to monitor d/tPRP target cell interactions. AP–d/tPRP was initially characterized by Western blot analysis. Samples were separated by polyacrylamide gel electrophoresis in 7.5% gels under reducing conditions and were electrophoretically transferred to nitrocellulose. Polyclonal antibodies generated against d/tPRP were used as probes. Immune complexes were detected using the enhanced chemiluminescence system. Lane A, recombinant d/tPRP; lane B, AP–d/tPRP preparations from HEK 293 cells stably transfected with the pCMV AP–d/tPRP expression vector; lane C, control conditioned medium from AP-transfected HEK 293 cells. Please note that the AP–d/tPRP fusion protein migrated at a  $M_r$  approximating 110 kDa, while native d/tPRP migrated at a  $M_r$  of 29.

PRL receptor. The AP–d/tPRP fusion protein bound readily to heparan sulfate-containing wild-type CHO cells but not to heparan sulfate-deficient CHO–pgsD–677 cells (Fig. 2, top panel). Moreover, AP–d/tPRP failed to bind heparan sulfate-deficient CHO cells transiently transfected with the long form of the rat PRL receptor (Fig. 2, bottom panel). In contrast, AP–PL–I, a known ligand of the PRL receptor, effectively bound to the PRL receptor transfected cells (Fig. 2, bottom panel). Neither d/tPRP or the AP–d/tPRP fusion protein stimulated the proliferation of the PRL-responsive Nb2 lymphoma cells (data not shown). Collectively, these observations are consistent with earlier reports that d/tPRP interacts with heparin-containing molecules and does not activate the PRL receptor signaling pathway (Rasmussen *et al.* 1996).

#### Identification of d/tPRP targets within the uterus

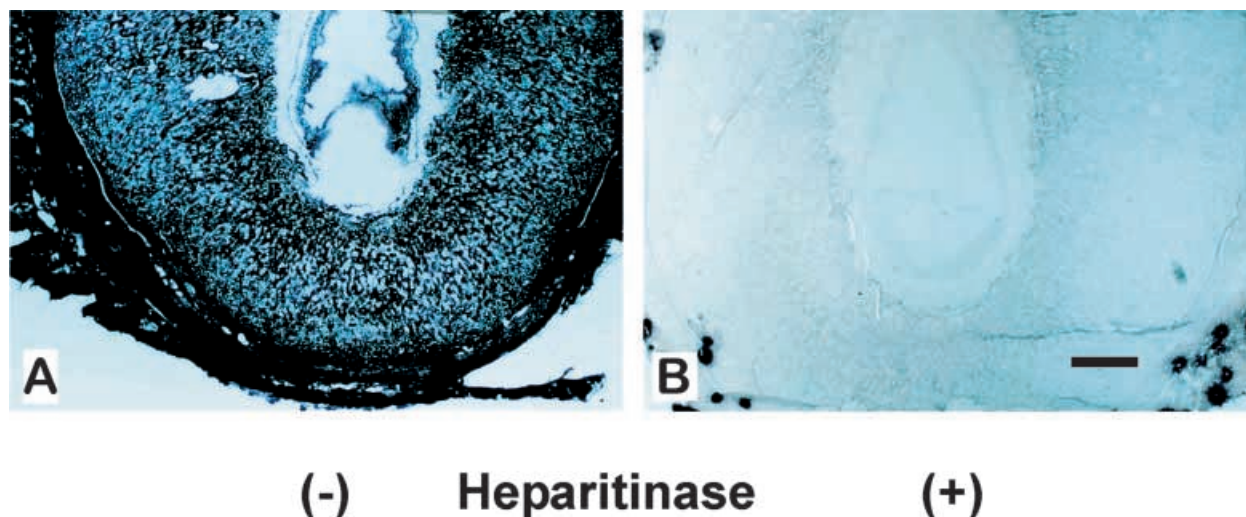
**Heparin-dependent interactions** AP–d/tPRP was used as a histo-specific marker to identify potential targets



**Figure 2** AP-d/tPRP binding to heparin-containing molecules and lack of binding to the PRL receptor. (Top panel) Wild-type CHO cells (A) and heparan sulfate-deficient CHO-pgsD-677 cells (B) were probed with the AP-d/tPRP fusion protein. Please note the extensive binding of AP-d/tPRP to the wild-type CHO heparan sulfate-containing cells and the relative absence of d/tPRP binding to the heparan sulfate-deficient CHO cells. The length of the bar in B represents 200  $\mu$ m. (Bottom panel) Heparan sulfate-deficient CHO-pgsD-677 cells were transiently transfected with the long form of the rat PRL receptor (pECE/long) and probed with the AP-d/tPRP (A and C) or AP-PL-I (B and D) fusion proteins. Please note that AP-d/tPRP failed to bind heparan sulfate-deficient CHO cells transiently transfected with the PRL receptor. In contrast, AP-PL-I bound to CHO cells transfected with the PRL receptor (PRLR). The length of the bar in B represents 200  $\mu$ m.

in mouse and rat uteroplacental tissues during various stages of gestation. The AP-d/tPRP probe bound to virtually all components of the uterus. This widespread interaction of AP-d/tPRP with uteroplacental compartments was shown to be dependent on heparin-containing molecules since pretreatment of tissue sections with heparitinase greatly decreased AP-d/tPRP binding

(Fig. 3) as did preincubation of AP-d/tPRP with heparin (data not shown). A population of non-heparin binding sites was identified by consecutively incubating tissue sections with AP-d/tPRP followed by excess heparin (250  $\mu$ g/ml to 10 mg/ml). This strategy led to the identification of discrete d/tPRP target cells within the endometrium and myometrium of the non-pregnant rat



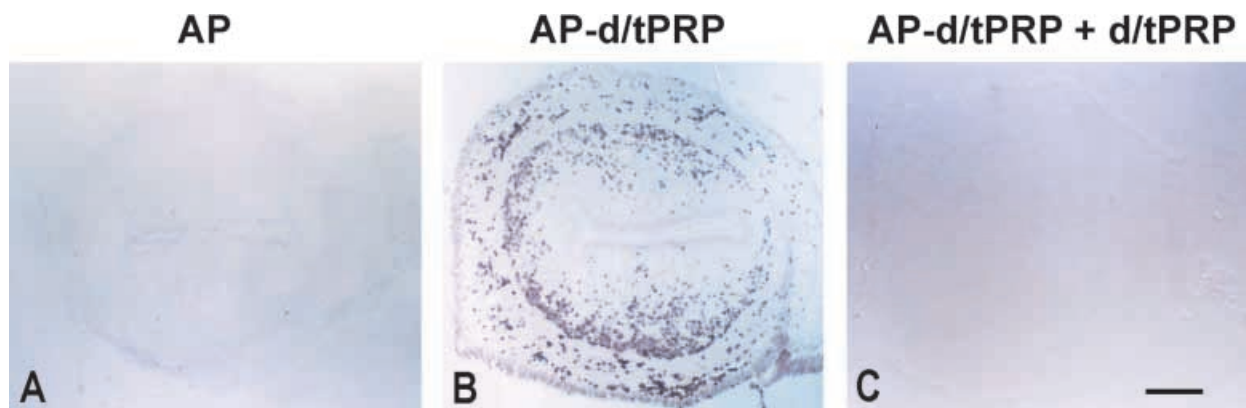
**Figure 3** AP-d/tPRP binding to heparin-containing molecules in the mouse uterus. AP-d/tPRP binding was detected by AP histochemistry. (A) Uterine tissue section from day 8 of pregnancy probed with AP-d/tPRP; (B) uterine tissue section from day 8 of pregnancy pretreated with heparitinase and probed with AP-d/tPRP. Please note that pretreatment of the tissue section with heparitinase significantly decreased AP-d/tPRP binding. The length of the bar in B represents 300  $\mu\text{m}$ .

uterus (Fig. 4). Specificity of the d/tPRP interaction with uterine cells was further demonstrated by use of an AP control probe, which showed no detectable binding to uterine tissue sections (Fig. 4) and by competition assays demonstrating that excess recombinant d/tPRP attenuated the binding of AP-d/tPRP to the uterine cells (Fig. 4).

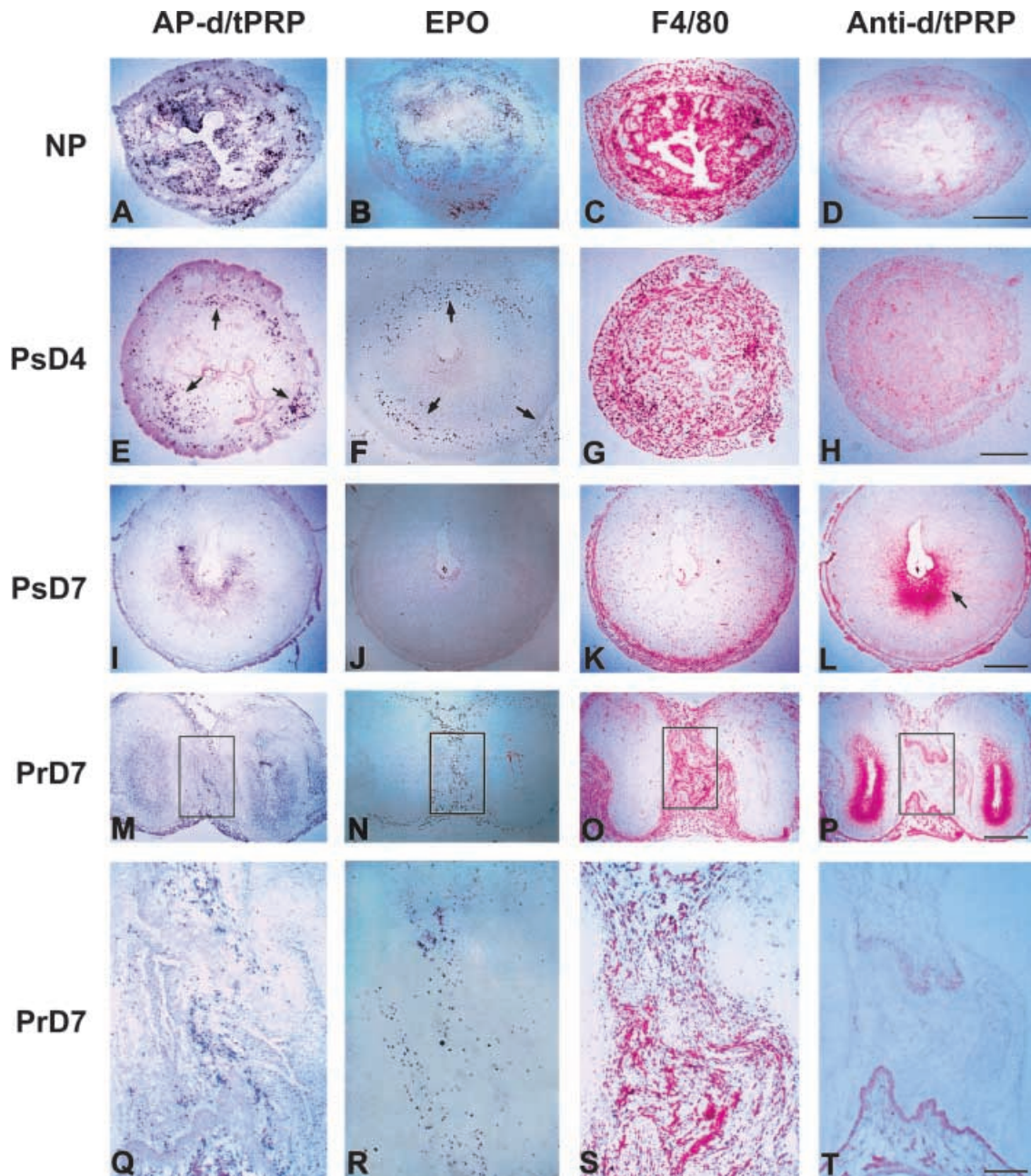
#### Distribution of d/tPRP binding within the uterus

The distribution of AP-d/tPRP target cells within mouse and rat uteri was similar to the distribution of leukocytes, especially macrophages and eosinophils within the uterus (Bassett 1962, Ross & Klebanoff 1966, Tachi & Tachi 1986, 1989, Redline *et al.* 1989, McMaster *et al.*

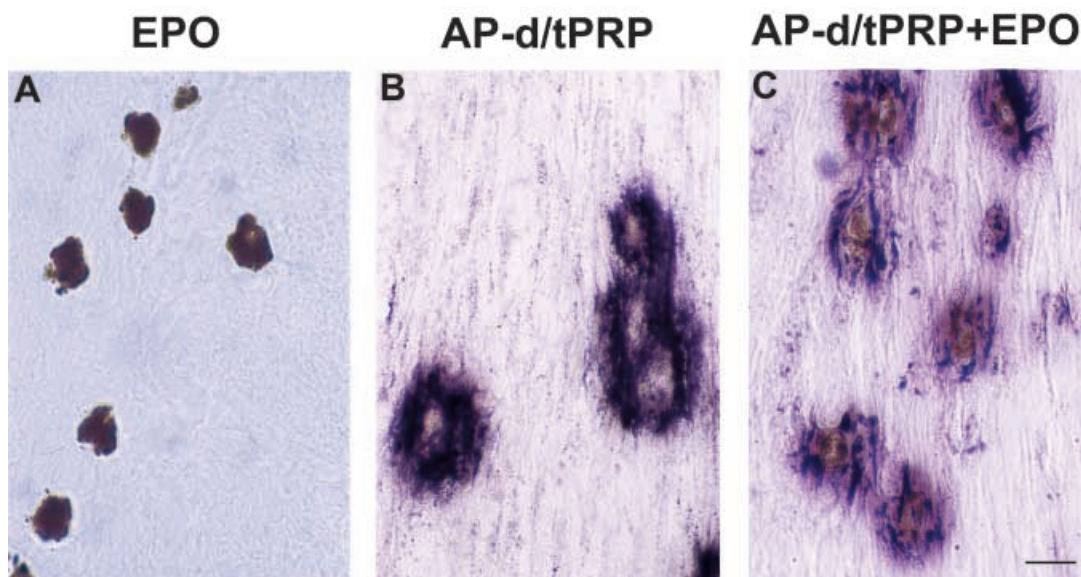
1992, King *et al.* 1981, Stewart & Mitchell 1991, 1992, Yelavarthi *et al.* 1991, Brandon 1993, 1995, Kaushic *et al.* 1998). In order to clarify this relationship, d/tPRP binding was compared with the distribution of the F4/80 antigen (a macrophage/eosinophil marker; Austyn & Gordon 1981, McGarry & Stewart 1991) and eosinophils via monitoring of eosinophil peroxidase histochemistry (King *et al.* 1981, Horton *et al.* 1996). In some cases, eosinophils were localized using immunocytochemistry for major basic protein-1, an eosinophil-specific granule protein (Dembele-Duchesne *et al.* 1991, Lee *et al.* 1997a). While the distributions of d/tPRP binding and F4/80 immunoreactivity were only roughly similar, the locations of



**Figure 4** AP-d/tPRP target cells in the uterus of the non-pregnant rat. Separation of AP-d/tPRP binding to heparan-containing molecules from other potential interactions was achieved by post-AP-d/tPRP incubation of tissue sections with heparin (250  $\mu\text{g}/\text{ml}$ ). AP-d/tPRP binding was detected by AP histochemistry. (A) Rat uterine tissue section probed with AP control; (B) rat uterine tissue section probed with AP-d/tPRP fusion protein; (C) rat uterine tissue section probed with AP-d/tPRP fusion protein excess recombinant d/tPRP. Please note that AP-d/tPRP binding to rat uterine tissue sections is specific. The length of the bar in C represents 250  $\mu\text{m}$ .



**Figure 5** Comparison of AP-d/tPRP binding, eosinophil distribution, F4/80 antigen distribution, and d/tPRP expression in uterine tissues from non-pregnant (NP), day 4 pseudopregnant (PsD4), day 7 pseudopregnant (PsD7), and day 7 pregnant (PrD7) mice. AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin (A, E, I, M and Q). Eosinophil distribution was detected by histochemistry for eosinophil peroxidase (EPO; B, F, J, N, and R). F4/80 antigen distribution was detected by immunocytochemistry (C, G, K, O and S). D/tPRP expression was monitored by immunocytochemistry with polyclonal antibodies to rat d/tPRP (D, H, L, P and T). (A–D) Uterine sections from a non-pregnant mouse; (E–H) uterine sections from a PsD4 mouse; (I–L) uterine sections from a PsD7 mouse with a deciduoma; (M–P) uterine sections containing two developing embryos and an interimplantation region; (Q–T) high magnification of the interimplantation segments shown in M–P. Note the abundance of AP-d/tPRP binding in uteri from non-pregnant mice, the relative exclusion of AP-d/tPRP binding in decidua associated with pseudopregnant and pregnant mice, and the reciprocal distribution of d/tPRP binding and d/tPRP expression in uteri PrD7 mice. Scale bars in D and H represent 500  $\mu$ m. A–H are photographed at the same magnification. Scale bars in L and P represent 1 mm. I–P are photographed at the same magnification. Scale bar in T is 250  $\mu$ m. Q–T are photographed at the same magnification. Arrows highlight the locations of the positive cells.



**Figure 6** Co-localization of AP-d/tPRP binding and eosinophil peroxidase in mouse uterine eosinophils. Eosinophil peroxidase (EPO) was detected by histochemistry (A). AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin (B). Dual localization of AP-d/tPRP binding and eosinophil peroxidase activity (C). Please note the 1:1 relationship between AP-d/tPRP binding and eosinophil peroxidase activity. The length of the bar in C is 10  $\mu$ m.

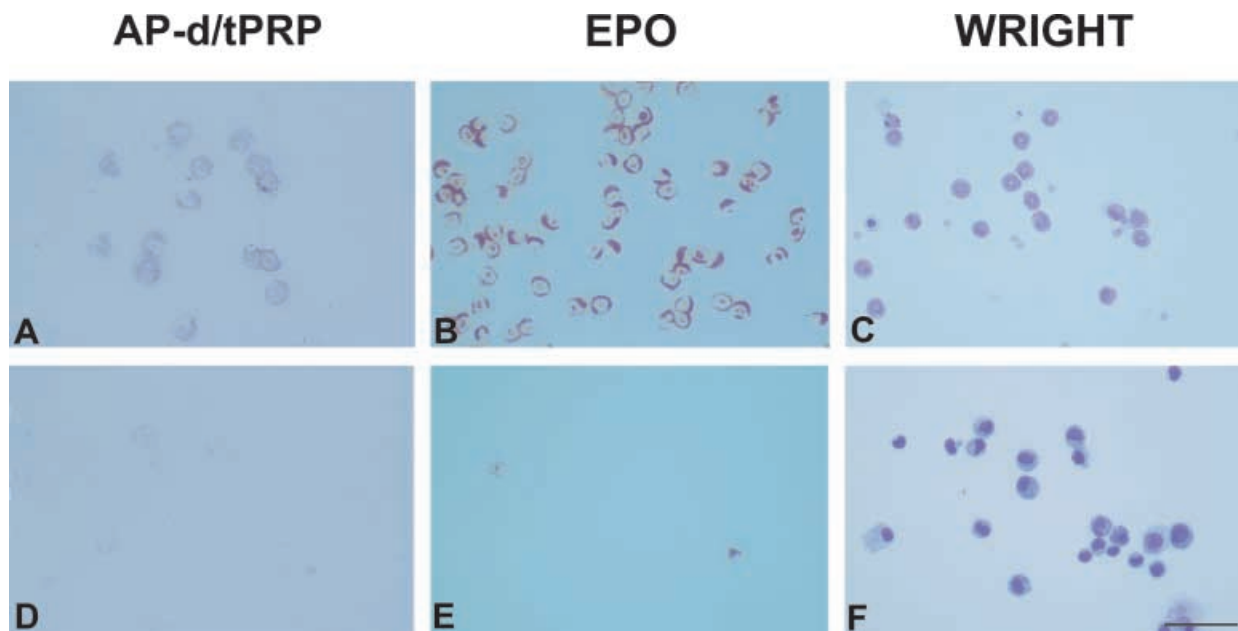
d/tPRP binding and eosinophils were virtually identical (Fig. 5). d/tPRP binding cells were most abundant in non-pregnant and day 4 pseudopregnant mouse endometrium and myometrium (Fig. 5A and E). Following the decidual reaction, d/tPRP binding cells and eosinophils were rare within mouse decidua (Fig. 5I, J, M, N, Q and R). d/tPRP binding was reciprocally related to the distribution of decidual cells expressing d/tPRP. Decidual cell patterns of d/tPRP expression were similar to previous reports from our laboratory (Fig. 5L and P; Orwig *et al.* 1997a, Rasmussen *et al.* 1997). During pregnancy in the mouse, F4/80 positive cells, d/tPRP binding cells and eosinophils were most prominent in interimplantation sites (Fig. 5Q, R and S). Furthermore, the number of F4/80 positive cells (macrophages+eosinophils) always outnumbered d/tPRP binding cells, especially in tissues from pseudopregnant and pregnant mice. These observations were consistent with d/tPRP interacting with eosinophils and/or a subpopulation of macrophages.

**Identification of eosinophils as d/tPRP targets** The relationship between d/tPRP binding cells, eosinophils and macrophages was further clarified by double labeling experiments. In addition, AP-d/tPRP binding to eosinophils and macrophages was examined using peritoneal cavity cells from the adult rat. Mouse uterine tissue sections dually processed for d/tPRP binding and the localization of eosinophil peroxidase showed that d/tPRP binding and eosinophil peroxidase co-localized (Fig. 6). In

contrast, mouse uterine tissue sections dually processed for d/tPRP binding and the immunolocalization of the F4/80 antigen showed that d/tPRP bound to only a small subset of F4/80 positive cells (data not shown). This F4/80 positive subpopulation may correspond to cells of the monocyte-macrophage lineage or the eosinophil granulocyte lineage (McGarry & Stewart 1991). Additional support for eosinophils as targets for d/tPRP was demonstrated via the specific binding of AP-d/tPRP to eosinophils isolated from the peritoneum (Fig. 7) but not to macrophages isolated from the peritoneum (Fig. 7). Collectively, the results are consistent with d/tPRP interacting with eosinophils.

The overall capacity of d/tPRP binding to eosinophils present in the rat or mouse uterus (Figs 4 and 5) was consistently greater than observed for eosinophils isolated from the peritoneum (Fig. 7) or blood (data not shown). These observations prompted an investigation of d/tPRP binding to eosinophils present in other tissues. d/tPRP bound avidly to eosinophils present in the rat intestine and in the rat lung (Fig. 8). Thus, d/tPRP binding is not unique to uterine eosinophils and appears to be enhanced by eosinophils situated within tissues.

The specific association of d/tPRP with tissue eosinophils was further demonstrated by examining AP-d/tPRP binding to tissues from normal and IL-5 transgenic mice (Lee *et al.* 1997b). IL-5 transgenic mice exhibit a generalized tissue eosinophilia. Uteri and lungs from prepubertal normal mice exhibit limited numbers of



**Figure 7** Binding of AP-d/tPRP to eosinophils versus macrophages. Eosinophils (A–C) and macrophages (D–F) were isolated from the rat peritoneum. AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin (A and D). Eosinophils were detected by eosinophil peroxidase (EPO) histochemistry (B and E). Total cells in the peritoneal eosinophil or macrophage preparations were detected with Wright stain (C and F). AP-d/tPRP bound to eosinophils but not macrophages. The length of the bar in F corresponds to 50  $\mu$ m. All micrographs were photographed at the same magnification.

eosinophils and d/tPRP binding cells (Fig. 9A and B, E and F), whereas these same tissues from IL-5 transgenic mice show extensive numbers of eosinophils and d/tPRP binding cells (Fig. 9C and D, G and H).

**Estrogen control of uterine d/tPRP binding and eosinophil distributions** Eosinophils exhibit dynamic changes within the uterus dependent upon the steroid hormone milieu. For example, estrogen stimulates the infiltration of eosinophils into the uterus (Bjersing & Borglin 1964, King *et al.* 1981, Tchernitchin 1983, Lee *et al.* 1989). This observation was confirmed by administration of exogenous estradiol (Fig. 10). Cells binding d/tPRP and possessing eosinophil peroxidase dramatically increased in uteri from the estradiol-treated versus the vehicle-treated control rats.

#### *Identification of d/tPRP targets within the chorioallantoic placenta*

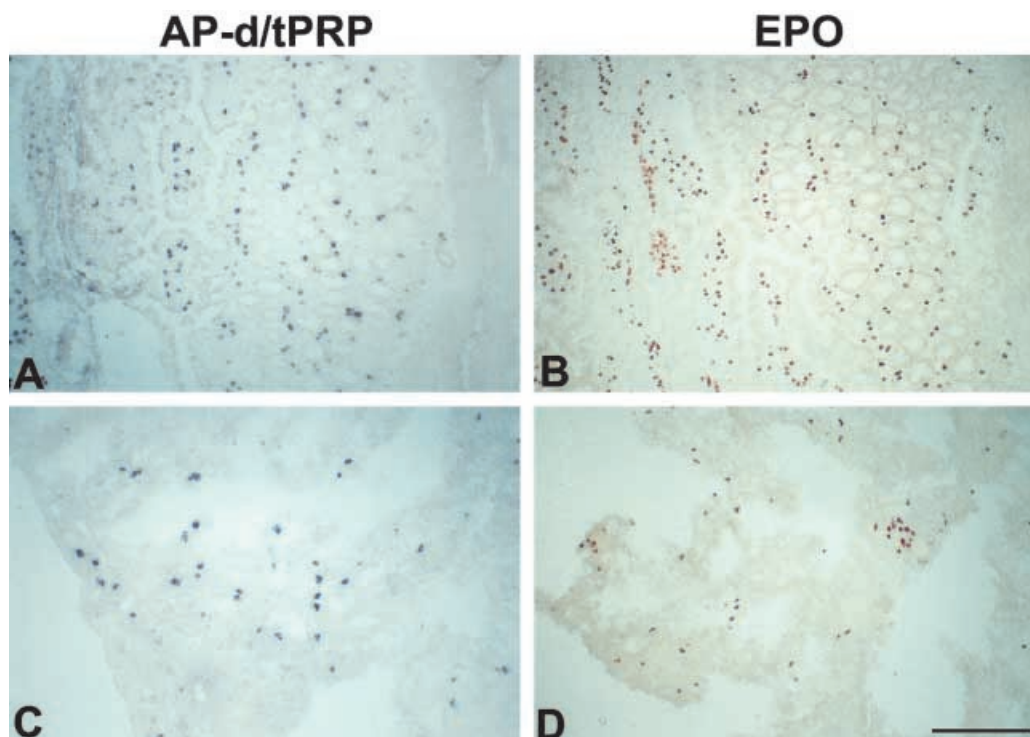
d/tPRP is characterized by its dual expression in decidual tissue from implantation until mid-gestation and in spongiotrophoblast and trophoblast giant cells of the chorioallantoic placenta from mid-gestation until the end of pregnancy (Orwig *et al.* 1997a,b; Rasmussen *et al.* 1997). AP-d/tPRP binding was examined in mouse and rat uteroplacental tissues from mid-pregnancy to term. AP-d/tPRP bound specifically and prominently to the

labyrinth zone of mouse (Fig. 11) and rat (data not shown) chorioallantoic placentas. d/tPRP binding appeared to increase as a function of gestational development.

## Discussion

### *d/tPRP interactions with heparin-containing molecules*

Heparin-containing molecules are prominently displayed on the external surfaces of cells and their extracellular matrices (Hassell *et al.* 1986). Several pieces of data are consistent with d/tPRP interactions with heparin-containing molecules: i) d/tPRP specifically binds to heparin Sepharose and is released from cultured cells by treatment with heparitinase (Rasmussen *et al.* 1996); ii) d/tPRP co-isolates with extracellular matrix proteins and is not detectable in maternal circulation (Rasmussen *et al.* 1996); iii) d/tPRP binds to wild-type heparan sulfate-containing CHO cells but not to heparan sulfate-deficient CHO-pgsD-677 cells (present study); iv) d/tPRP binding to tissues is dramatically affected by pretreatment with heparitinase and/or heparin (present study). d/tPRP-heparin interactions are likely relevant to the actions of d/tPRP on its targets. These ligand-heparin associations presumably facilitate the accumulation of d/tPRP in proximal locations and prevent d/tPRP from accessing systemic targets. d/tPRP interactions with heparin may also contribute conformationally to the biological

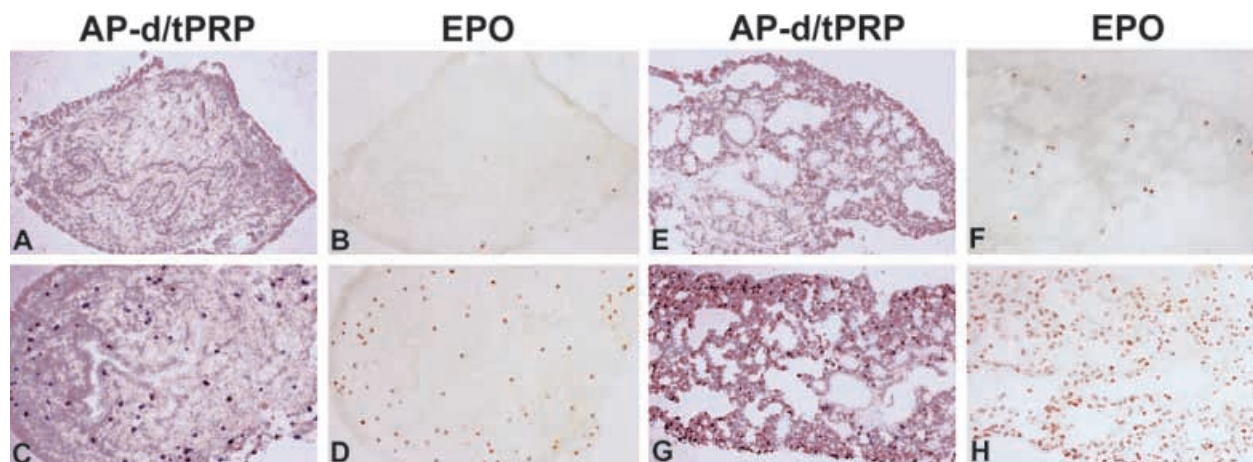


**Figure 8** AP-d/tPRP binding and eosinophils distributions in rat intestinal (A and B) and rat lung tissues (C and D). AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin (A and C). Eosinophils were detected by eosinophil peroxidase (EPO) histochemistry (B and D). The length of the bar in D corresponds to 200  $\mu$ m. All micrographs were photographed at the same magnification.

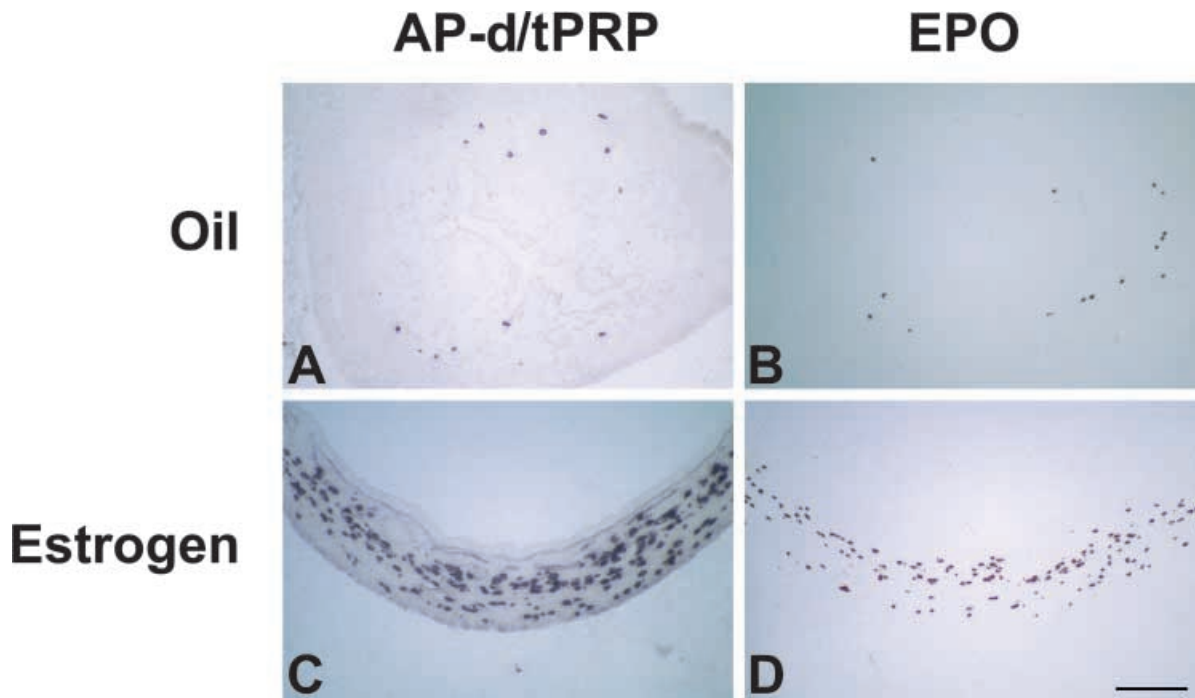
activation of d/tPRP and/or the prevention of d/tPRP degradation.

Diffusion of d/tPRP away from its cellular sources within decidua versus the chorioallanotic placenta likely

differ due to differences in the heparan sulfate composition of the two sites. Extracellular matrix of the decidua is richly endowed in heparan sulfate proteoglycans (Wewer *et al.* 1986), whereas the junctional zone of the



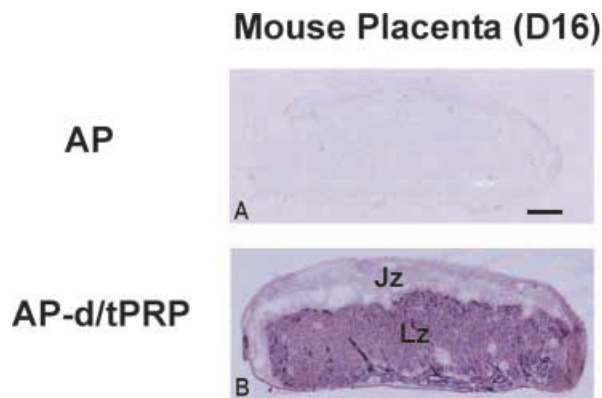
**Figure 9** AP-d/tPRP binding and eosinophil distributions in tissues from normal (A and B; E and F) and IL-5 transgenic mice (C and D; G and H). Analyses were performed on uterine (A–D) and lung (E–G) tissues from prepubertal female mice. AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin (A, C, E and G). Eosinophils were detected by eosinophil peroxidase (EPO) histochemistry (B, D, F and H). All micrographs were photographed at the same magnification.



**Figure 10** Estrogen control of uterine d/tPRP binding and eosinophil distributions. Prepubertal rats (day 20 postnatal) were subcutaneously injected daily for 3 days with estradiol valerate (100 µg/100 µl; A and B) or vehicle (sesame oil; C and D). Twenty-four hours following the last injection, rats were killed, uterine tissues dissected and frozen for subsequent analysis. AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin (A and C). Eosinophils were detected by eosinophil peroxidase histochemistry (B and D). The length of the bar in D corresponds to 100 µm. All micrographs were photographed at the same magnification.

chorioallantoic placenta is conspicuously devoid of heparan sulfate proteoglycans (Laurie 1985). This dichotomy in the environment surrounding the sites of d/tPRP synthesis presumably results in differential access to targets within these tissues. During the first half of pregnancy, d/tPRP

diffusion is likely restricted to the decidual cells responsible for its synthesis and the associated extracellular matrix, whereas during the second half of gestation, d/tPRP is likely capable of diffusing to tissues bordering the junctional zone, i.e. decidual basalis and labyrinth zone of the chorioallantoic placenta.



**Figure 11** AP-d/tPRP binding to the mouse chorioallantoic placenta isolated from gestation day 16. AP-d/tPRP binding was detected by AP histochemistry following a postincubation with heparin. (A) AP control; (B) AP-d/tPRP. Please note that AP-d/tPRP specifically bound to the labyrinth zone of the chorioallantoic placenta. The length of the bar in A represents 500 µm.

#### *d/tPRP interactions with eosinophils*

Eosinophils are components of inflammatory responses that are likely to participate in immune surveillance against parasitic infections and solid tumors (Gleich *et al.* 1993, Venge 1993, Weller 1993, Tepper *et al.* 1992, Tepper 1994, Hirai *et al.* 1997, Rothenberg 1998, Giembycz & Lindsay 1999, LeMoine *et al.* 1999*a,b*). These cells are identified based on the composition of their granules which contain a variety of cytotoxic molecules that can cause considerable damage to surrounding normal cells (Gleich *et al.* 1993, Venge 1993, Weller 1993, Hirai *et al.* 1997, Rothenberg 1998, Giembycz & Lindsay 1999).

Eosinophils are normal, and relatively abundant, constituents of both rodent and human uterine endometria (Rytomaa 1960, Bassett 1962, Ross & Klebanoff 1966, McMaster *et al.* 1992, Jeziorska *et al.* 1995). In rodents, the most dramatic changes in uterine eosinophil distributions

occur prior to mating and during pregnancy. Eosinophils infiltrate into the uterine endometrium preceding mating and are effectively removed from the vicinity of the developing embryo accompanying the establishment of pregnancy (McMaster *et al.* 1992, present study). It may be advantageous to have eosinophils present in the uterus around the time of mating when there is a possibility for the introduction of potential infectious agents. Likewise, it would also appear efficacious to remove or restrain the eosinophils once the pregnancy has been initiated in order to prevent their attack of the semi-allogeneic embryo. Mechanisms underlying this form of immunoprotection have not been well defined. Decidual tissue represents a barrier effectively excluding eosinophils (present study). Progesterone participates in the control of the development of decidual cells (DeFeo 1967, Lydon *et al.* 1995) and possesses key anti-inflammatory actions within the uterus (Siiteri & Stites 1982, Finn 1986, Tibbetts *et al.* 1999). d/tPRP likely contributes to the maternal adaptation to pregnancy and may mediate, at least in part, the anti-inflammatory actions of progesterone. Once secreted, d/tPRP is trapped in the decidual extracellular matrix where it associates with heparin-containing molecules and has access to migratory cells, including eosinophils (Rasmussen *et al.* 1996, present study). Intracellular pathways activated by d/tPRP may culminate in the protection of the developing embryo from the potentially cytotoxic actions of eosinophils. In this hypothesis, alteration of eosinophil activities facilitates survival of genetically disparate extraembryonic and embryonic tissues. d/tPRP is capable of interacting with eosinophils located in uterine and extrauterine tissues (present study) and with eosinophils from other species (including guinea pig, dog and cow; D Wang, D Walia & M J Soares, unpublished results). Systemic effects of d/tPRP on extrauterine eosinophils are likely obviated by the association of d/tPRP with heparin-containing molecules on the cell surface and within the extracellular matrix.

#### *d/tPRP interactions with the labyrinthine placental compartment*

As gestation progresses, d/tPRP is produced in the junctional zone of the chorioallantoic placenta (Rasmussen *et al.* 1997), where it presumably diffuses to adjoining tissues due to the absence of heparan sulfate proteoglycan within the junctional zone (Laurie 1985). This secreted d/tPRP may potentially target the underlying labyrinthine trophoblast and eosinophils in the decidual basalis (present study).

#### *Overview*

d/tPRP is part of a larger family of regulatory molecules that help ensure viviparity. This family includes: PRL, which possesses specific modulatory actions on the

maternal ovaries, liver, and mammary gland (Soares *et al.* 1998); proliferin and proliferin-related protein, which influence development of vasculature within the uteroplacental compartment (Jackson *et al.* 1994); PRL-like protein-A, which specifically targets uterine natural killer cells (Müller *et al.* 1999); and PRL-like protein-E, which controls aspects of hematopoiesis (Lin & Linzer 1999). The biology of other members of the uteroplacental PRL family are yet to be described.

The nature of d/tPRP's actions on eosinophils or the labyrinthine placenta is not known. d/tPRP does not interact with the PRL receptor (Rasmussen *et al.* 1996, present study). Consequently, d/tPRP actions are likely mediated by alternative signaling pathways. It is not entirely clear whether specific and unique receptor signaling systems have co-evolved with each PRL family member, whether PRL family members utilize signaling pathways for other known ligands, or whether PRL family members act through receptor-independent mechanisms (e.g. transport proteins, binding proteins, etc.).

In summary, using an AP tagging strategy, d/tPRP is shown to specifically interact with eosinophils and the labyrinthine placenta. We propose that d/tPRP participates in the regulation of uterine eosinophils and the labyrinthine placenta in ways that contribute to the establishment and maintenance of pregnancy.

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#### **References**

- Ali S, Edery M, Pellegrin I, Lesueur L, Paly J, Djiane J & Kelly PA 1992 The Nb2 form of prolactin receptor is able to activate a milk protein gene promoter. *Molecular Endocrinology* **6** 1242-1248.
- Austyn JM & Gordon S 1981 F4/80: a monoclonal antibody directed specifically against the mouse macrophage. *European Journal of Immunology* **11** 805-811.

- Bassett EG 1962 Infiltration of eosinophils into the modified connective tissue of oestrous and pregnant animals. *Nature* **194** 1259–1261.
- Bell SC 1983 Decidualization: regional differentiation and associated function. *Oxford Review of Reproductive Biology* **5** 220–271.
- Berger J, Hauber J, Hauber R, Geiger R & Cullen BR 1988 Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **66** 101–106.
- Bjersing L & Borglin NE 1964 Effect of hormones on incidence of uterine eosinophilia in rats. *Acta Pathologica Microbiologica Scandinavica* **60** 353–364.
- Brandon JM 1993 Leucocyte distribution in the uterus during the preimplantation period of pregnancy and phagocyte recruitment to sites of blastocyst attachment in mice. *Journal of Reproduction and Fertility* **98** 567–576.
- Brandon JM 1995 Macrophage distribution in decidual tissue from early implantation to the periparturient period in mice as defined by the macrophage differentiation antigens F4/80, macrosialin and the type 3 complement receptor. *Journal of Reproduction and Fertility* **103** 9–16.
- Deb S, Hamlin GP, Roby KF, Kwok SCM & Soares MJ 1993 Heterologous expression and characterization of prolactin-like protein-A: identification of serum binding proteins. *Journal of Biological Chemistry* **268** 3298–3305.
- DeFeo VJ 1967 Decidualization. In *Cellular Biology of the Uterus*, pp 191–290. Ed. RM Wynn. New York: Appleton-Century-Crofts.
- Dembele-Duchesne M-J, Badia E, Etienne-Julian M & Capony J-P 1991 Identification and tissue localization of an eosinophil 17 kDa protein accumulating in rat uterus upon estradiol treatment. *Journal of Steroid Biochemistry and Molecular Biology* **38** 321–330.
- Dijkstra CD, Dopp EA, Joling P & Kraal G 1985 The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2, and ED3. *Immunology* **54** 589–599.
- Dileepan KN, Lorsch RB & Stechschulte DJ 1993 Mast cell granules inhibit macrophage-mediated lysis of mastocytoma cells (P815) and nitric oxide production. *Journal of Leukocyte Biology* **53** 446–453.
- Enders AC & Welsh AO 1993 Structural interactions of trophoblast and uterus during hemochorial placenta formation. *Journal of Experimental Zoology* **266** 578–587.
- Finn CA 1986 Implantation, menstruation and inflammation. *Biological Reviews* **61** 313–328.
- Giembycz MA & Lindsay MA 1999 Pharmacology of the eosinophil. *Pharmacological Reviews* **51** 213–339.
- Gleich GJ, Adolphson CR & Leiferman KM 1993 The biology of the eosinophilic leukocyte. *Annual Review of Medicine* **44** 85–101.
- Gu Y, Soares MJ, Srivastava RK & Gibori G 1994 Expression of decidual prolactin-related protein in the rat decidua. *Endocrinology* **135** 1422–1427.
- Hassell JR, Kimura JH & Hascall VC 1986 Proteoglycan core protein families. *Annual Review of Biochemistry* **55** 539–567.
- Hirai K, Miyamasu M, Takaishi T & Morita Y 1997 Regulation of the function of eosinophils and basophils. *Critical Reviews in Immunology* **17** 325–352.
- Horton MA, Larson KA, Lee JJ & Lee NA 1996 Cloning of the murine eosinophil peroxidase gene (mEPO): characterization of a conserved subgroup of mammalian hematopoietic peroxidases. *Journal of Leukocyte Biology* **60** 285–294.
- Howe RS, Lee YH, Fischkoff SA, Teuscher C & Lyttle CR 1990 Glucocorticoid and progesterin regulation of eosinophil chemotactic factor and complement C3 in the estrogen-treated rat uterus. *Endocrinology* **126** 3193–3199.
- Jackson D, Volpert OV, Bouck N & Linzer DIH 1994 Stimulation and inhibition of angiogenesis by placental proliferin and proliferin-related protein. *Science* **266** 1581–1584.
- Jeziorska M, Salamonsen LA & Woolley DE 1995 Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. *Biology of Reproduction* **53** 312–320.
- Kausch K, Frauendorf E, Rossoll RM, Richardson JM & Wira CR 1998 Influence of the estrous cycle on the presence and distribution of immune cells in the rat reproductive tract. *American Journal of Reproductive Immunology* **39** 209–216.
- Kay AB, Barata L, Meng Q, Durham SR & Ying S 1997 Eosinophils and eosinophil-associated cytokines in allergic inflammation. *International Archives of Allergy and Immunology* **113** 196–199.
- King WJ, Allen TC & DeSombre ER 1981 Localization of uterine peroxidase activity in estrogen-treated rats. *Biology of Reproduction* **25** 859–870.
- Laurie GW 1985 Lack of heparan sulfate proteoglycan in a discontinuous and irregular placental basement membrane. *Developmental Biology* **108** 299–309.
- Lee JJ, McGarry MP, Farmer SC, Denzler KL, Larson KA, Carrigan PE, Brenneise IE, Horton MA, Haczu A, Gelfand EW, Leikauf GD & Lee NA 1997a Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *Journal of Experimental Medicine* **185** 2143–2156.
- Lee NA, McGarry MP, Larson KA, Horton MA, Kristensen AB & Lee JJ 1997b Expression of IL-5 in thymocytes/T cells leads to the development of massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *Journal of Immunology* **158** 1332–1344.
- Lee YH, Howe RS, Sha S-J, Teuscher C, Sheehan DM & Lyttle CR 1989 Estrogen regulation of an eosinophil chemotactic factor in the immature rat uterus. *Endocrinology* **125** 3022–3028.
- Le Moine A, Flamand V, Demoor F-X, Noel J-C, Surquin M, Kiss R, Nahori M-A, Pretolani M, Goldman M & Abramowicz D 1999a Critical roles for IL-4, IL-5, and eosinophils in chronic skin allograft rejection. *Journal of Clinical Investigation* **103** 1659–1667.
- Le Moine A, Surquin M, Demoor F-X, Noel JC, Nahori M-A, Pretolani M, Flamand V, Braun MY, Goldman M & Abramowicz D 1999b IL-5 mediates eosinophilic rejection of MHC class II-disparate skin allografts in mice. *Journal of Immunology* **163** 3778–3784.
- Lidholt K, Weinke JL, Kiser CS, Lugenwa FN, Bame KJ, Cheifetz S, Massague J, Lindahl U & Esko JD 1992 A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *PNAS* **89** 2267–2271.
- Lin J & Linzer DIH 1999 Induction of megakaryocyte differentiation by a novel pregnancy-specific hormone. *Journal of Biological Chemistry* **274** 21485–21489.
- Lin J, Poole J & Linzer DIH 1997 Three new members of the mouse prolactin/growth hormone family are homologous to proteins expressed in the rat. *Endocrinology* **138** 5541–5549.
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM & O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes and Development* **9** 2266–2278.
- McGarry MP & Stewart CC 1991 Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. *Journal of Leukocyte Biology* **50** 471–478.
- MacKenzie CD, Jungery M, Taylor PM & Ogilvie BM 1981 The *in vitro* interaction of eosinophils, neutrophils, macrophages and mast cells with nematode surfaces in the presence of complement or antibodies. *Journal of Pathology* **133** 161–175.
- McMaster MT, Newton RC, Dey SK & Andrews GK 1992 Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period. *Journal of Immunology* **148** 1699–1705.
- Müller H, Dai G & Soares MJ 1998 Placental lactogen-I (PL-I) target tissues identified with an alkaline phosphatase-PL-I fusion protein. *Journal of Histochemistry and Cytochemistry* **46** 737–744.

- Müller H, Liu B, Croy BA, Head JR, Dai G & Soares MJ 1999 Uterine natural killer cells are targets for a trophoblast cell-specific cytokine, prolactin-like protein-A. *Endocrinology* **140** 2711–2720.
- Orwig KE, Dai G, Rasmussen CA & Soares MJ 1997a Decidual/trophoblast prolactin related protein: characterization of gene structure and cell-specific expression. *Endocrinology* **138** 2491–2500.
- Orwig KE, Ishimura R, Müller H, Liu B & Soares MJ 1997b Identification and characterization of a mouse homolog for decidual/trophoblast prolactin-related protein. *Endocrinology* **139** 5511–5517.
- Parr MB & Parr EL 1989 The implantation reaction. In *Biology of the Uterus*, pp 233–278. Eds RM Wynn & WP Jollie. New York: Plenum.
- Pearlman E 1997 Immunopathology of onchocerciasis: a role for eosinophils in onchocercal dermatitis and keratitis. *Chemical Immunology* **66** 26–40.
- Rasmussen CA, Hashizume K, Orwig KE, Xu L & Soares MJ 1996 Decidual prolactin-related protein: heterologous expression and characterization. *Endocrinology* **137** 5558–5566.
- Rasmussen CA, Orwig KE, Vellucci S & Soares MJ 1997 Dual expression of prolactin-related protein in decidua and trophoblast tissues during pregnancy. *Biology of Reproduction* **55** 647–654.
- Redline RW & Lu CY 1989 Localization of fetal major histocompatibility complex antigens and maternal leukocytes in murine placenta. *Laboratory Investigation* **61** 27–36.
- Roby KF, Deb S, Gibori G, Szpirer C, Levan G, Kwok SCM & Soares MJ 1993 Decidual prolactin-related protein: identification, molecular cloning, and characterization. *Journal of Biological Chemistry* **268** 3136–3142.
- Ross R & Klebanoff SJ 1966 The eosinophilic leukocyte. Fine structure studies of changes in the uterus during the estrous cycle. *Journal of Experimental Medicine* **124** 653–659.
- Rothenberg ME 1998 Eosinophilia. *New England Journal of Medicine* **338** 1592–1600.
- Rytomaa T 1960 Organ distribution and histochemical properties of eosinophil granulocytes in rat. *Acta Pathologica Microbiologica Scandinavica* (Suppl) **140** 11–118.
- Siiteri P & Stites DP 1982 Immunologic and endocrine interrelationships in pregnancy. *Biology of Reproduction* **26** 1–14.
- Soares MJ 1987 Developmental changes in the intraplacental distribution of placental lactogen and alkaline phosphatase in the rat. *Journal of Reproduction and Fertility* **79** 93–98.
- Soares MJ, Colosi P, Ogren L & Talamantes F 1983 Identification and partial characterization of a lactogen from the midpregnant mouse conceptus. *Endocrinology* **112** 1313–1317.
- Soares MJ, Dai G, Orwig KE, Peters TJ & Müller H 1998 The uteroplacental prolactin family and pregnancy. *Biology of Reproduction* **58** 273–284.
- Stewart IJ & Mitchell BS 1991 The distribution of uterine macrophages in virgin and early pregnant mice. *Journal of Anatomy* **179** 183–196.
- Stewart IJ & Mitchell BS 1992 Macrophages and other endocytic cells in the mouse uterus during the second half of pregnancy and into the postpartum period. *Journal of Anatomy* **181** 119–126.
- Tachi C & Tachi S 1986 Macrophages and implantation. *Annals of the New York Academy of Sciences* **476** 158–182.
- Tachi C & Tachi S 1989 Role of macrophages in the maternal recognition of pregnancy. *Journal of Reproduction and Fertility* **37** 63–68.
- Tanaka T, Shiu RPC, Gout PW, Beer CT, Noble RL & Friesen HG 1980 A new sensitive and specific bioassay for lactogenic hormones: measurement of prolactin and growth hormone in human serum. *Journal of Clinical Endocrinology and Metabolism* **51** 1058–1062.
- Tchernitchin AN 1983 Eosinophil-mediated non-genomic parameters of estrogen stimulation – a separate group of responses mediated by an independent mechanism. *Journal of Steroid Biochemistry* **19** 95–100.
- Tepper RI 1994 The eosinophil-mediated antitumor activity of interleukin-4. *Journal of Allergy and Clinical Immunology* **94** 1225–1231.
- Tepper RI, Coffman RL & Leder P 1992 An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science* **257** 548–551.
- Tibbetts TA, Conneely OM & O'Malley BW 1999 Progesterone via its receptor antagonizes the pro-inflammatory activity of estrogen in the mouse uterus. *Biology of Reproduction* **60** 1158–1165.
- Venge P 1993 Human eosinophil granule proteins: structure, function, and release. In *Immunopharmacology of eosinophils*, pp 43–56. Eds H Smith & RM Cook. New York: Academic Press.
- Weller PF 1993 Lipid, peptide, and cytokine mediators elaborated by eosinophils. In *Immunopharmacology of eosinophils*, pp 25–42. Eds H Smith & RM Cook. New York: Academic Press.
- Wewer UM, Damjanov A, Weiss J, Liotta LA & Damjanov I 1986 Mouse endometrial stromal cells produce basement-membrane components. *Differentiation* **32** 49–58.
- Yelavarthi KK, Chen H-L, Yang Y, Cowley BD, Fishback JL & Hunt JS 1991 Tumor necrosis factor- $\alpha$  mRNA and protein in rat uterine and placental cells. *Journal of Immunology* **146** 3840–3848.

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