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A uterine decidual cell cytokine ensures pregnancy-dependent adaptations to a physiological stressor

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In the mouse, decidual cells differentiate from uterine stromal cells in response to steroid hormones and signals arising from the embryo. Decidual cells are crucially involved in creating the intrauterine environment conducive to embryonic development. Among their many functions is the production of cytokines related to prolactin (PRL), including decidual prolactin-related protein (DPRP). DPRP is a heparin-binding cytokine, which is abundantly expressed in uterine decidua. In this investigation, we have isolated the mouse *Dprp* gene, characterized its structure and evaluated its biological role. *Dprp*-null mice were made by replacing exons 2 to 6 of the *Dprp* gene with an in-frame enhanced green fluorescent protein (*EGFP*) gene and a neomycin (*neo*) resistance cassette. Heterozygous intercross breeding of the mutant mice yielded the expected mendelian ratio. Pregnant heterozygote females expressed EGFP within decidual tissue in locations identical to endogenous *Dprp* mRNA and protein expression. Homozygous *Dprp*-null mutant male and female mice were viable, exhibited normal postnatal growth rates, were fertile and produced normal litter sizes. A prominent phenotype was observed when pregnant *Dprp*-null mice were exposed to a physiological stressor. DPRP deficiency interfered with pregnancy-dependent adaptations to hypoxia resulting in pregnancy failure. Termination of pregnancy was associated with aberrations in mesometrial decidual cells, mesometrial vascular integrity, and disruptions in chorioallantoic placenta morphogenesis. The observations suggest that DPRP participates in pregnancy-dependent adaptations to a physiological stressor.

KEY WORDS: *Dprp* (*Dtprp*), Decidua, Pregnancy, Uterus, Null mutation, Adaptations to hypoxia, Mouse

INTRODUCTION

The establishment of pregnancy requires maternal adjustments. Hemochorial placentation, which occurs in both primates and rodents, results in the establishment of a close connection between maternal and fetal tissues (Enders and Welsh, 1993; Carson et al., 2000). This close connection facilitates the exchange of nutrients and wastes. Decidual and trophoblast cells are likely to provide the signaling system that coordinates the activities of the maternal compartment. Decidual cells are modified uterine endometrial stromal cells. The differentiation of decidual cells is one of the earliest uterine adaptations to pregnancy (DeFeo, 1967; Parr and Parr, 1989; Aplin, 2000). Decidual cell differentiation is exquisitely sensitive to the regulatory actions of progesterone, interleukin-11, and activators of cyclic AMP/protein kinase A (Tang et al., 1994; Lydon et al., 1995; Brar et al., 1997; Dimitriadis et al., 2005; Brosens and Gellersen, 2006). During gestation, decidual cells are located at the interface separating invading trophoblast cells from the maternal environment. A number of important functions have been attributed to decidua (Bell, 1983; Aplin, 2000; Brosens and Gellersen, 2006): (1) a protective role in controlling trophoblast cell invasion; (2) a nutritive role for the developing embryo; (3) a role in preventing immunological rejection of genetically disparate embryonic/fetal tissues; and (4) an endocrine/paracrine role in controlling maternal adaptations required for the establishment and maintenance of pregnancy. Pregnancy is dependent upon decidual cell acquisition of each of these specialized functions. Disruptions in decidual cell development are not compatible with pregnancy (Lydon et al., 1995; Bilinski et al., 1998; Robb et al., 1998; Mantena et al., 2006). Progress in understanding specialized decidual cell functions has been limited.

Decidual cell signaling is mediated, at least in part, through the production of cytokines related to prolactin (PRL) (Tang et al., 1994; Orwig et al., 1997c; Telgmann and Gellersen, 1998; Jabbour and Critchley, 2001). PRL is a member of a larger collection of structurally-related hormones/cytokines (the PRL superfamily) with an array of different biological targets and actions (Wiemers et al., 2003; Soares, 2004; Alam et al., 2006). In the rat and mouse, four members of the PRL superfamily are expressed in uterine decidua: decidual prolactin-related protein (DPRP; DTPRP – Mouse Genome Informatics) (Roby et al., 1993; Lin et al., 1997; Orwig et al., 1997b), prolactin-like protein B (PLP-B; PRLPB – Mouse Genome Informatics) (Duckworth et al., 1988; Croze et al., 1990; Cohick et al., 1997; Müller et al., 1998), PLP-J (PRLPJ – Mouse Genome Informatics) (Hiraoka et al., 1999; Ishibashi and Imai, 1999; Toft and Linzer, 1999; Dai et al., 2000) and prolactin itself (Prigent-Tessier et al., 1999; Kimura et al., 2001). Each of these decidual PRL family cytokines can be viewed as a downstream mediator of intrauterine progesterone action.

DPRP is secreted as a glycoprotein by uterine decidual cells and resides in the decidual extracellular matrix where it binds with high affinity to heparin-containing molecules (Rasmussen et al., 1996; Rasmussen et al., 1997; Orwig et al., 1997b; Wang et al., 2000). Little is known about the physiological actions of DPRP. In this report, we explore the biology of uterine decidual cells through investigation of the *Dprp*-null mouse.

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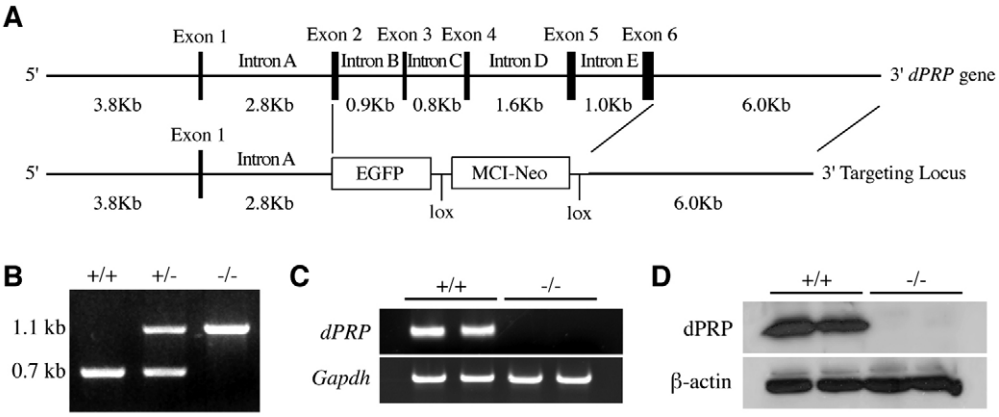

Fig. 1. Mouse *Dprp* gene, construction of a *Dprp*-null mutant targeting vector, genotype analysis, and *Dprp* mRNA and protein expression. (**A**) Exons 2-6 of the mouse *Dprp* gene were replaced with an in-frame *EGFP* gene followed by an *MC1neo* cassette. (**B**) PCR analysis of wild-type (+/+), heterozygous (+/–) and null (–/–) alleles. (**C**) RT-PCR analysis of *Dprp* transcripts in gestation day 7.5 decidua from wild-type (+/+) and *Dprp*-null (–/–) mice. (**D**) Western blot analysis of DPRP protein in gestation day 7.5 decidua from wild-type (+/+) and *Dprp*-null (–/–) mice.

MATERIALS AND METHODS

Gene targeting

A genomic DNA library generated from a 129/SvEv strain mouse liver and packaged in the Lambda FIX II vector was a generous gift of Lexicon Genetics (Houston, TX). Approximately 1×10^6 pfu were screened with a mouse *Dprp* cDNA (Orwig et al., 1997b). Positive plaques were amplified and used to inoculate LE392 *Escherichia coli.* A series of forward and reverse oligonucleotide primer sets based on the mouse *Dprp* cDNA were designed and used to sequence exons and exon-intron boundaries. DNA sequencing was performed with an Applied Biosystems Model 310 sequencer and Applied Biosystems Dye Terminator Cycle Sequencing Kits (Foster City, CA). The *Dprp* targeting vector was constructed by replacing exons 2-6 of the mouse *Dprp* gene with the enhanced green fluorescent protein (*EGFP*) gene and *MC1neo* cassette flanked by *loxP* sites (Godwin et al., 1998). A 6.6 kb DNA fragment, containing 3.8 kb of 5′ flanking DNA and 2.8 kb of exon 1 and intron A of the mouse *Dprp* genomic construct, was subcloned upstream of *EGFP*. A 6.0 kb DNA fragment of the *Dprp* genomic construct containing 3′ flanking DNA located immediately downstream of exon 6 was subcloned downstream of the *MC1neo* cassette and upstream of a herpes simplex virus thymidine kinase gene. The accuracy of vector construction was verified by restriction enzyme and DNA sequence analyses. A schematic representation of the mouse *Dprp* gene and the targeting vector are shown in Fig. 1. The targeting vector was introduced into R1 embryonic stem cells (Nagy et al., 1993) (a generous gift from Dr Janet Rossant, Samuel Lunenfeld Research Institute, Toronto, Canada) by electroporation. Cells were selected by exposure to G418 and gangcyclovir. Southern blot analysis was used to identify clones that appropriately underwent homologous recombination with the targeting vector. Genomic DNA was isolated, digested with *Sal*I, and fractionated in 0.8% agarose gels. Southern blots were performed with a probe derived from intron A. Wild-type alleles were characterized by a 21 kb hybridization signal; homozygous mutant alleles were characterized by a 5.5 kb hybridization signal. Chimeras were generated by injection into C57BL/6 blastocysts and transferred into pseudopregnant (C57BL/6×CBA) F1 females. PCR was routinely used to identify offspring with wild-type and *Dprp* mutant alleles. A forward primer corresponding to a nucleotide sequence in intron A of the *Dprp* gene (5′-GAGCTTAAACTTCAATGTAAGT-3′) was used with reverse primers corresponding to nucleotide sequences in intron B of the *Dprp* gene (5′-GTGTGCTAAATGAACGTAGT-3′) and within the *EGFP* gene (5′-GTATGGCTGATTATGATCTAGA-3′). PCR was conducted for 30 cycles under the following conditions: preheat, 94°C for 4 minutes; denature, 94°C for 1 minute; anneal, 60°C for 1 minute; and extension, 72°C for 1.5 minutes. PCR products (wild-type allele, 676 bp; mutant allele, 1148 bp) were separated on 1% agarose gels and stained with Ethidium Bromide. Mice with the *Dprp* mutation were backcrossed for six generations to C57BL/6 or 129SvJ genetic backgrounds.

Animals and tissue preparation

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were housed in an environmentally controlled facility, with lights on from 0600-2000 h, and allowed free access to food and water. Timed matings of animals were conducted by placing females with fertile males. The day when a seminal plug was found in the vagina of female mice was designated as day 0.5 of pregnancy. Placentation sites, including uterus, decidual, and placental tissues, were dissected from pregnant animals. Pseudopregnancy was induced by mating with vasectomized males. Deciduomal reactions were induced on day 3.5 of pseudopregnancy by injecting 25 μl of sesame oil/uterine horn. Harvested tissues were snap-frozen in liquid nitrogen for RNA and protein analyses. For in situ hybridization and immunohistochemical analyses, tissues were frozen in dry ice-cooled heptane. All tissue samples were stored at –80°C until used. Protocols for the above procedures have been described (Deb et al., 2006; Ain et al., 2006). Alkaline phosphatase activities in deciduomal tissue were measured as previously described (Soares, 1987; Arroyo et al., 2005). The University of Kansas Medical Center Animal Care and Use Committee approved all procedures for handling and experimentation with rodents.

Hypobaric hypoxia

Female C57BL/6 pregnant mice were placed in hypobaric chambers beginning on day 5.5 of gestation, as previously described (Ho-Chen et al., 2006). Under these conditions, air is circulated at a barometric pressure of

Table 1. Primer sets used for analysis of decidual PRL family transcripts

Gene	Forward primer	Reverse primer
Dprp	5′-TGAATGTCAAACAGGAGAGAA-3′	5′-CAATCTTGCCCAGTTATGCGG-3′
Plp-j	5′-TATGACCGGAAATCCAATGAA-3′	5′-GGTTTTGATTTTGCCATGCTT-3′
Plp-b	5′-GCACTTCAAAAGCCTGATACT-3′	5′-GTGCCCATGTATGCCAGTTTG-3′
Gapdh	5′-ACCACACTCCATGCCATCAC-3′	5′-TCCACCACCCTGTTGCTGTA-3′

Table 2. Genotypic distribution of offspring from heterozygous matings

Strains	+/+	+/–	–/–	Male:Female
Mixed	54	105	47	106:100
C57BL/6	64	109	56	119:110
129/SvJ	60	106	57	107:116

Heterozygous breedings yielded the expected mendelian ratio (1:2:1). Male:female ratio of wild-type (+/+), heterozygous (+/–) and *Dprp*-null mutant (–/–) mice were also comparable to each other.

Table 4. Reproductive performance of *Dprp* mutant mice

Strains	Breeding combinations		
	+/+ × +/+	+/– × +/–	–/– × –/–
Mixed	7.92±2.35 (*n*=12)	9.08±2.19 (*n*=12)	7.83±1.59 (*n*=12)
C57BL/6	8.71±1.58 (*n*=21)	8.24±2.53 (*n*=21)	8.66±1.8 (*n*=16)
129/SvJ	6.6±1.69 (*n*=16)	6.41±1.47 (*n*=22)	7.27±1.18 (*n*=18)

Values are expressed as the mean±s.d.

~420 Torr, which results in an inspired PO_2 of ~78 Torr, equivalent to breathing 11% O_2 at sea level. The chambers were opened daily to clean cages and replenish food and water (15-20 minutes).

Phenotypic analyses of the uteroplacental compartment

Western blot analysis

DPRP protein was detected in tissue extracts by immunoblotting as previously described (Rasmussen et al., 1996; Orwig et al., 1997b). Protein concentrations were determined for each sample using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA).

Histological analyses

Analyses were performed on 10 μm tissue sections prepared with the aid of a cryostat. Sections were stained with Hematoxylin and Eosin, or subjected to biotinylated *Griffonia simplicifolia* lectin I isolectin B_4 (Vector Laboratories, Peterborough, UK) histochemistry, or used for immunocytochemistry. Immunocytochemical analyses were used to determine the distribution of GFP, natural killer (NK) cells, trophoblast cells and endothelial cells (Ain et al., 2003) (T.K., L. A. Rempel, J. A. Arroyo and M.J.S., unpublished). GFP was monitored by fluorescence and immunoreactivity with rabbit anti-GFP polyclonal antibodies (Chemicon International, Temecula, CA). NK cells were detected with a rabbit polyclonal anti-perforin 1 antibody (Torrey Pines Biolabs, Houston, TX). Trophoblast cells were monitored with a rat monoclonal anti-mouse cytokeratin antibody (TROMA-1; Developmental Studies Hybridoma Repository, Iowa City, IA). Endothelial cells were localized using a rat monoclonal anti-mouse endoglin antibody (Developmental Studies Hybridoma Repository, Iowa City, IA) and a rat monoclonal anti-mouse CD31 antibody (BD Pharmingen, Franklin Lakes, NJ). TUNEL assays were performed with the In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. All processed tissue sections were examined and images recorded with a Leica MZFLIII stereomicroscope equipped with a CCD camera (Leica Microsystems GmbH, Welzlar, Germany).

PRL superfamily mini-array assay

The PRL superfamily mini-array assay is a hybridization-based tool for simultaneously monitoring expression of each member of the PRL superfamily (Dai et al., 2002). The assay has been effectively used to monitor the phenotypes of decidua and placenta. The PRL superfamily mini-array assay was performed as previously described (Dai et al., 2002).

Northern blot analysis

Northern blot analysis was performed as described previously (Faria et al., 1990). Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (15 μg per lane) was resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes and crosslinked. Blots were probed with [α^{32}P]-labeled cDNAs for *Dprp* (Orwig et al., 1997b), *Plp-j* (Dai et al., 2000), *Plp-b* (Müller et al., 1998) and metallothionein-I (*Mt1*) (Liang et al., 1996). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) cDNA was used to evaluate the integrity and equal loading of RNA samples. At least three different tissue samples from three different animals were analyzed with each probe for each time point.

RT-PCR analysis

Dprp, *Plp-j* and *Plp-b* mRNA levels were estimated by RT-PCR. Total RNA was isolated from uterine tissues from days 5.5 to 7.5 of gestation. Total RNA (2 μg) and 0.5 μg of oligo dT were used for reverse transcription reactions with SuperScript II reverse transcriptase (Invitrogen). PCR was conducted using Platinium Taq DNA High Fidelity polymerase (Invitrogen) and *Dprp*-, *Plp-j*-, *Plp-b*- or *Gapdh*-specific primers (Table 1). PCR was performed for 30 cycles (denature, 95°C for 45 seconds; anneal, 55°C for 45 seconds; extension, 72°C for 1 minute). The amplified products were resolved by electrophoresis in 1% agarose gels and Ethidium Bromide staining.

In situ hybridization

The localization of mRNAs within tissues was performed as described previously (Ain et al., 2003; Weimers et al., 2003). Cryosections (10 μm) of tissues were prepared and stored at –80°C until used. Plasmids containing cDNAs for mouse *Dprp* and *Plp-j* (Orwig et al., 1997b; Dai et al., 2000) were used as templates to synthesize sense and antisense digoxigenin-labeled riboprobes according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN).

Statistical analysis

The data were analyzed by analysis of variance and post hoc comparisons determined by the Newman-Keuls Test.

RESULTS

Generation of a *Dprp*-null mouse

Screening of a mouse genomic library with the mouse *Dprp* cDNA resulted in the isolation of a phage clone containing the entire coding sequence for mouse *Dprp*. The *Dprp* gene possesses a 6-exon organization, similar to rat *Dprp* and other members of the PLP-C (PRLPC – Mouse Genome Informatics) subfamily (Dai et al., 1996; Orwig et al., 1997a; Wiemers et al., 2003; Alam et al., 2006). *Dprp*-null mutant mice were generated by gene-targeting strategies culminating in the replacement of a region of

Table 3. Body weight at 55 days of age

Strains		+/+	+/–	–/–
Mixed	Male	29.02±2.67 (*n*=36)	28.54±3.80 (*n*=100)	28.49±4.06 (*n*=61)
	Female	22.17±2.90 (*n*=48)	22.17±3.04 (*n*=82)	21.41±3.32 (*n*=54)
C57BL/6	Male	24.67±1.74 (*n*=27)	24.83±2.09 (*n*=47)	23.57±1.75 (*n*=26)
	Female	18.18±1.56 (*n*=22)	18.63±1.11 (*n*=42)	18.63±1.74 (*n*=27)
129/SvJ	Male	25.26±2.21 (*n*=20)	24.67±2.63 (*n*=43)	23.65±1.61 (*n*=21)
	Female	20.65±1.82 (*n*=24)	19.76±2.21 (*n*=53)	18.98±1.44 (*n*=28)

There are no significant differences in body weight (g) among the male or female wild-type (+/+), heterozygous (+/–) or *Dprp*-null mutant (–/–) mice. Values are expressed as the mean±s.d.

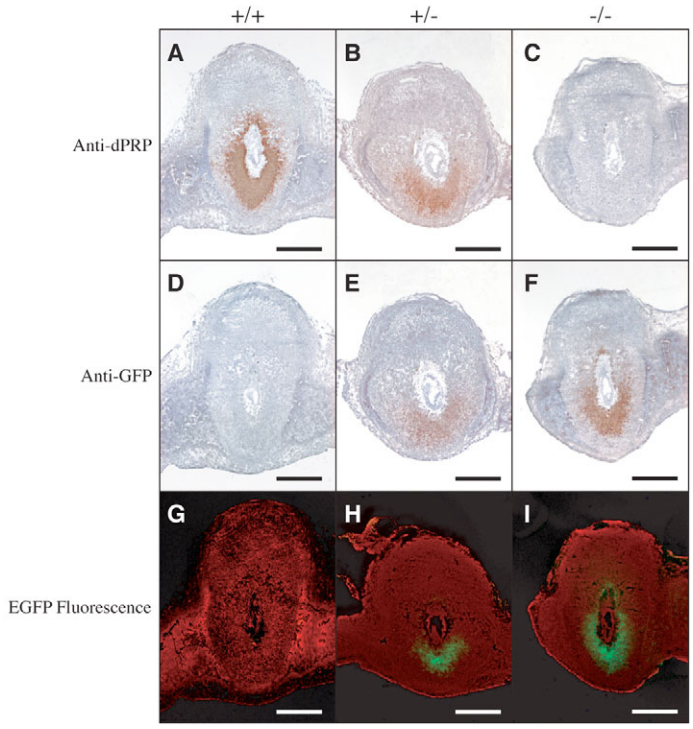

Fig. 2. *Dprp* and *Dprp*^{GFP} allele expression in the uteroplacental compartment in implantation sites of wild-type, heterozygous and *Dprp*-null mutant mice. Immunostaining for DPRP (**A-C**) and GFP (**D-F**) was performed on frozen sections from gestation day 7.5 implantation sites of wild-type (A,D), heterozygous (B,E) and homozygous mutant (C,F) mice. (**G-I**) EGFP fluorescence is shown in sections from gestation day 7.5 implantation sites of wild-type (G), heterozygous mutant (H) and homozygous mutant (I) mice (counterstain, Propidium Iodide). The mesometrial region of the uterus is located at the top of each image. Scale bars: 1 mm.

the *Dprp* gene (exons 2 through 6) with an in-frame *EGFP* gene and an *MC1neo* cassette. The portion of the *Dprp* coding sequence remaining in the mutated gene (exon 1) encodes the first 10 amino acids of the DPRP signal peptide. A schematic representation of the mouse *Dprp* gene and the targeting vector are shown in Fig. 1. Correct homologous recombination was determined by Southern blotting and PCR analyses. Two mutant ES cell lines (No. 44 and No. 96) with a normal karyotype were injected into blastocysts in order to generate chimeras. The No. 44 cell line gave rise to a >95% male chimera. The No. 96 cell line gave rise to three chimeras, including two males of 40% and 75% chimerism, and a female of 60% chimerism. Male chimeras from both the No. 44 and No. 96 lines were bred to C57BL/6 females and successfully transmitted the *Dprp* mutant allele to their offspring. Subsequent analyses were derived from mouse line No. 44. Breeding of mice heterozygous for the *Dprp*-null mutation resulted in offspring genotypes that did not significantly deviate from the expected mendelian ratio (Table 2). The mutation was moved to two inbred strains (C57BL/6 and 129SvJ) following six generations of backcrosses. Homozygous *Dprp*-null mutant male and female mice were viable on a mixed 129 SvJ and C57BL/6 genetic background and following transfer to C57BL/6 and 129SvJ genetic backgrounds. The offspring exhibited normal postnatal growth rates and were fertile (Tables 3 and 4). Genetic background did not significantly affect the phenotype of mice with the *Dprp*-null mutation. Genotyping and *Dprp* expression analyses are shown in Fig. 1. The gene targeting strategy successfully disrupted *Dprp* mRNA and protein expression.

Characterization of the uterine compartment in *Dprp* mutant mice

DPRP is known to be expressed in decidual and deciduomal tissues from both pregnant and pseudopregnant animals (Rasmussen et al., 1996; Rasmussen et al., 1997; Lin et al., 1997; Orwig et al., 1997b). The *Dprp*-null allele contains an EGFP gene inserted into the *Dprp* locus. Pregnant heterozygous (+/–) and homozygous null (–/–) females faithfully expressed EGFP within decidual tissue in locations similar to endogenous DPRP expression in pregnant wild-type (+/+) females (Fig. 2). However, the tissue distribution of EGFP in the mesometrial compartment of *Dprp*-null mice was less than the tissue distribution of DPRP protein in the mesometrial compartment of wild-type mice (Fig. 2). This difference continued to be evident on day 11.5 of gestation (Fig. 3).

Pregnancy proceeded in the absence of detectable *Dprp* mRNA. DPRP deficiency influenced the expression of another member of the decidual PRL superfamily, *Plp-j* (Fig. 4A). *Plp-j* mRNA levels were decreased in *Dprp*-null mutant decidua. DPRP deficiency did not significantly affect the expression of two other decidual products, *Plp-b* and *Mt1* (Fig. 4A), and did not significantly affect expression of other members of the PRL superfamily within the placenta on days 12.5 or 17.5 of gestation (Fig. 4B).

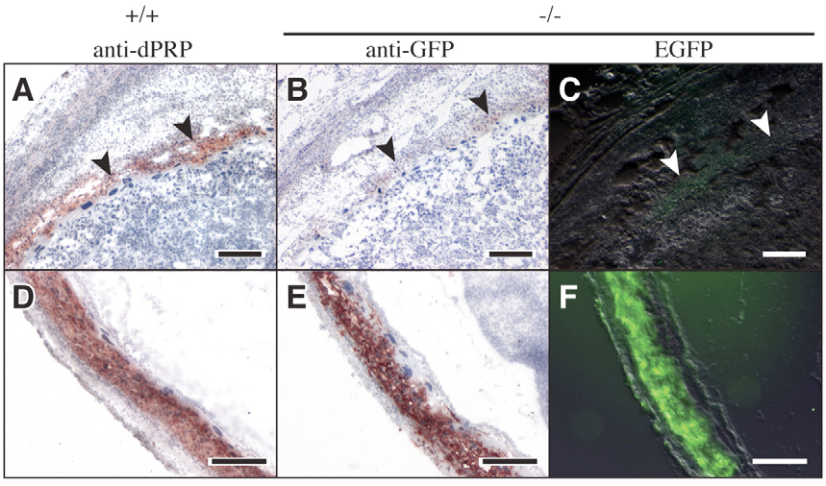

Fig. 3. Histological examination of mesometrial and anti-mesometrial decidua of gestation day 11.5 wild-type and *Dprp*-null mice. Mesometrial (**A-C**) and anti-mesometrial (**D-F**) decidua were monitored for DPRP expression in wild-type (+/+) tissues (A,D), and for GFP in DPRP-null (–/–) tissues using anti-GFP (B,E) and fluorescence (C,F). The arrowheads in A-C indicate the location of the mesometrial decidua. Note the minimal GFP expression in the mesometrial decidua of B and C. By contrast, the anti-mesometrial decidual regions appear comparable in wild-type and *Dprp*-null tissues. Scale bars: 250 μm.

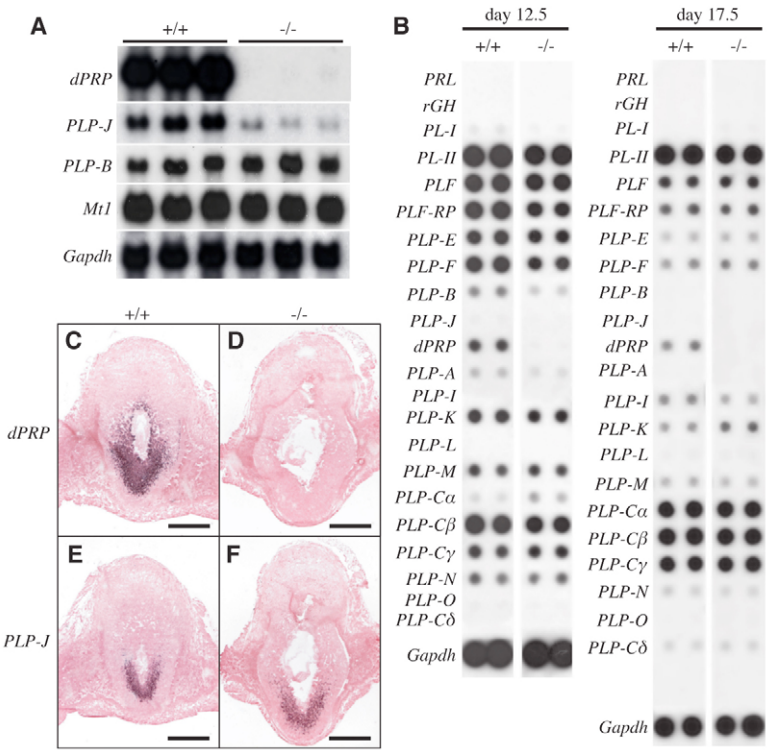

Fig. 4. Expression analysis of wild-type and *Dprp*-null mice. (**A**) Northern analysis for *Dprp*, *Plp-j*, *Plp-b* and *Mt1* in decidual tissues. Total RNA was isolated from decidual tissues of wild-type (+/+) and *Dprp*-null (–/–) mice on day 7.5 of gestation. *Gapdh* was used to demonstrate integrity of the RNA and loading accuracy. (**B**) PRL superfamily expression patterns were examined in mouse placentas using the PRL superfamily miniarray assay. cDNAs for all members of the mouse PRL superfamily were spotted on to nylon membranes. Total RNA from day 12.5 or day 17.5 placental tissues were used to make probes by reverse-transcription. *Gapdh* and salmon sperm DNA were used as controls. (**C-F**) Localization of *Dprp* (C,D) and *Plp-j* (E,F) mRNAs in implantation sites of wild-type (+/+; C,E) and *Dprp*-null (–/–; D,F) mice on day 7.5 of gestation. *Dprp* and *Plp-j* plasmids were used as templates for the synthesis of digoxigenin-labeled sense and anti-sense RNA probes. The sense probes did not demonstrate specific staining (data not shown). The mesometrial region of the uterus is located at the top of each image. Scale bars: 1 mm.

We next examined decidualization in pseudopregnant wild-type and *Dprp*-null mice (Fig. 5). Deciduoma formation was similar in mice of both genotypes with only subtle differences, including a modest but significant decrease in deciduomal weight, when expressed per body weight (Fig. 5D). DPRP protein in wild-type and GFP in *Dprp*-null mice localized predominantly to the anti-mesometrial deciduomal compartment (Fig. 5F-H). Similar to pregnancy, *Plp-j* mRNA expression was also down-regulated in *Dprp*-null deciduoma (Fig. 5I).

The organization of the maternal-fetal interface was examined. Distributions of endothelial (endoglin and CD31) and NK cell (perforin 1) markers and TUNEL activity did not differ between wild-type and *Dprp*-null uteroplacental compartments on gestation days 7.5 and 9.5 (data not shown).

Overall, the DPRP deficiency appeared to have only modest consequences for the establishment and maintenance of pregnancy and the organization of the maternal-fetal interface under standard husbandry conditions.

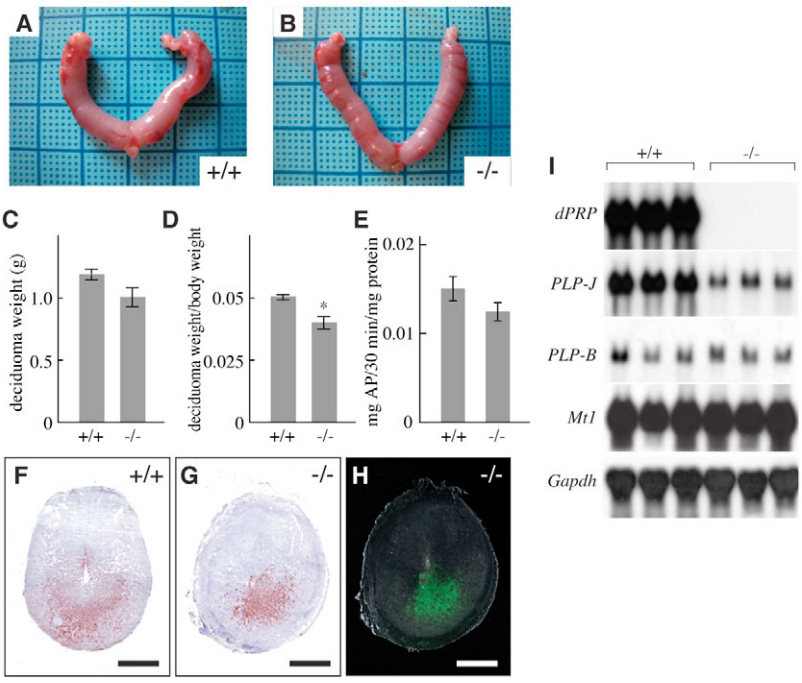

Fig. 5. Decidualization responses in wild-type and *Dprp*-null mice. (**A**,**B**) Gross appearance of artificially decidualized uteri from day 7.5 pseudopregnant wild-type (+/+) and *Dprp*-null (–/–) mice. (**C**) Day 7.5 pseudopregnant deciduoma weight responses from wild-type (+/+; *n*=7) and *Dprp*-null (–/–; *n*=9) mice. (**D**) Day 7.5 pseudopregnant deciduoma weight responses from wild-type (+/+; *n*=7) and *Dprp*-null (–/–; *n*=9) mice expressed by ratio to body weight. *, $P<0.01$. (**E**) Alkaline phosphatase (AP) activities of day 7.5 pseudopregnant deciduoma from wild-type (+/+; *n*=7) and *Dprp*-null (–/–; *n*=7) mice. (**F**) Immunocytochemical localization of DPRP in the day 7.5 pseudopregnant-decidualized uterus from wild-type (+/+) mice. (**G**) Immunocytochemical localization of GFP in the day 7.5 pseudopregnant-decidualized uterus from *Dprp*-null (–/–) mice. (**H**) GFP fluorescence in the day 7.5 pseudopregnant-decidualized uterus from *Dprp*-null (–/–) mice. The mesometrial region of the uterus is located at the top of each image. (**I**) Northern blot analysis of *Dprp*, *Plp-j*, *Plp-b*, *Mt1* and *Gapdh* expression in deciduoma from day 7.5 pseudopregnant wild-type (+/+) and *Dprp*-null (–/–) mice. Scale bars: 1 mm.

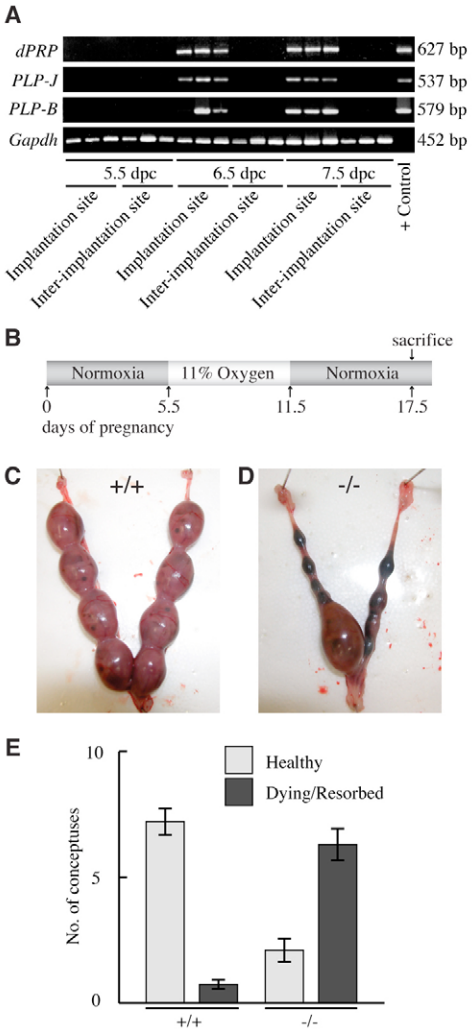

Fig. 6. Pregnancies in *Dprp*-null mice are vulnerable to maternal hypoxia. (**A**) Determination of the ontogeny of decidual PRL family expression by RT-PCR analysis. (**B**) Exposure of pregnant females to hypobaric hypoxia (equivalent of 11% oxygen) from days 5.5 to 11.5 of gestation. After day 11.5, the animals were returned to ambient conditions and examined on day 17.5 of gestation. (**C**,**D**) Gross appearance of a representative uterus from pregnant wild-type (+/+) and *Dprp*-null (–/–) mice exposed to hypoxia. (**E**) Quantification of pregnancy outcomes in wild-type (+/+; n=19) and *Dprp*-null mutant (–/–; n=10) mice exposed to hypoxia. Numbers of healthy and dying/resorbed conceptuses are significantly different between wild-type and *Dprp*-null mutant pregnancies; P<0.01. Note that unlike wild-type pregnant female mice, *Dprp*-null pregnant female mice do not adapt effectively to hypoxia.

Impact of maternal hypoxia on the *Dprp*-null phenotype

Successful species develop strategies to optimize their reproductive performance. This optimization is likely to include the evolution of genes that specifically permit reproduction in physiologically challenging conditions. The PRL superfamily has been postulated to participate in the regulation of adaptations to physiological stressors (Dorshkind and Horseman, 2001; Ain et al., 2004; Soares et al., 2006). These insights led us to examine a role for DPRP in the regulation of pregnancy-dependent adaptations to physiological stressors. Hypoxia was selected as a physiological stressor because it is well established that low oxygen tension promotes extensive tissue remodeling at the maternal-fetal interface (Zamudio, 2003; Fryer and Simon, 2006; Myatt, 2006).

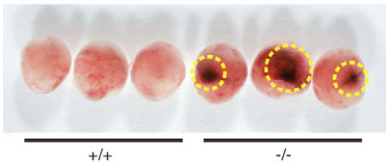

Fig. 7. Gross inspection of decidua-placental compartments from wild-type and *Dprp*-null pregnant mice exposed to hypobaric hypoxia. Wild-type (+/+) and *Dprp*-null (–/–) pregnant mice were exposed to the equivalent of 11% oxygen from days 5.5 to 11.5 of gestation. Mice were sacrificed on day 11.5 of gestation and uteroplacental compartments were dissected. The locations of hemorrhagic regions are encircled (yellow broken line) within the dissected uteroplacental compartments. Note the presence of prominent hemorrhagic areas in the *Dprp*-null (–/–) tissues.

In order to determine the time course for the physiological challenge, we first examined the ontogeny of decidual PRL family (*Dprp*, *Plp-j* and *Plp-b*) gene expression. *Dprp* expression was initiated between days 5.5 and 6.5 of gestation (Fig. 6A). Consequently, we challenged pregnant wild-type and *Dprp*-null mice from days 5.5 to 11.5 (duration of decidual *Dprp* expression during normal pregnancy) with the equivalent of 11% oxygen (21% oxygen is ambient at sea level). After hypoxia exposure, animals were returned to ambient conditions and examined on day 17.5 of gestation (Fig. 6B). Pregnant female mice possessing the mutant *Dprp* gene did not adapt to hypoxia as well as did wild-type mice (Fig. 6C-E). Most fetal-placental units were healthy on day 17.5 of gestation in wild-type animals; whereas most fetal-placental units were dying or resorbing in the *Dprp*-null mice. We conclude from these observations that DPRP participates in pregnancy-dependent adaptations to hypoxia.

Analysis of the maternal-fetal interface in *Dprp*-null mice exposed to hypoxia

The defects responsible for pregnancy termination in *Dprp*-null mice exposed to hypoxia were unique. Initial gross inspection and histological examination indicated that only modest effects were evident by day 9.5 of gestation in *Dprp*-null mice exposed to hypoxia. However, notable pathologies were identified by day 11.5 of gestation; macroscopic lesions were discernible in the mesometrial region or in the mesometrial-anti-mesometrial junction of *Dprp*-null uteroplacental compartments, but were not evident in wild-type uteroplacental compartments (Fig. 7). Maternal hypoxia did not significantly affect decidual *Dprp* gene expression in wild-type mice or decidual EGFP expression in *Dprp*-null mice (data not shown).

Histological examination of tissue sections through the uteroplacental compartments revealed prominent adaptive as well as a range of potentially maladaptive responses to hypoxia in the wild-type and *Dprp*-null mice (Figs 8, 9). The adaptive responses to maternal hypoxia observed in the wild-type uterine mesometrial compartment included compression of the mesometrial decidua and increased depth of endovascular trophoblast cell invasion. The potentially maladaptive responses in the *Dprp*-null mesometrial compartment included: (1) enlarged mesometrial blood spaces (Fig. 8A,D); (2) distorted chorioallantoic placental organization, including trophoblast giant cell overgrowth (Fig. 8B,E); (3) exaggerated compression of the mesometrial decidua (Fig. 8C,F);

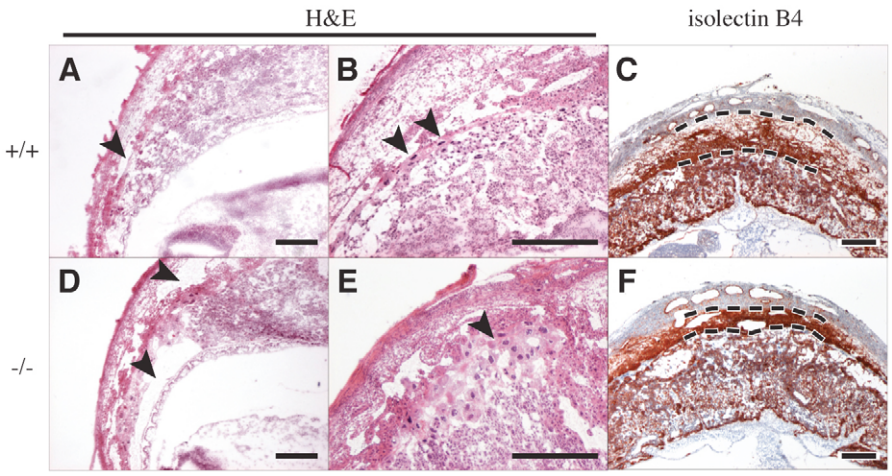

Fig. 8. Histological examination of day 11.5 uteroplacental compartments of wild-type and *Dprp*-null mice exposed to hypobaric hypoxia. (**A-C**) Wild-type (+/+) and (**D-F**) *Dprp*-null (–/–) mice were exposed to hypobaric hypoxia. Tissue sections were stained with Hematoxylin and Eosin (A,B,D,E) or by isolectin B_4 histochemistry (C,F). Note the enlarged mesometrial blood spaces (A versus D, arrowheads), the overgrowth of trophoblast giant cells (B versus E, arrowheads), and the compressed mesometrial decidua and enlarged chorioallantoic placenta (C versus F, dashed black lines demarcate the thickness of the mesometrial decidual layer) in the *Dprp*-null tissues. The mesometrial region of the uterus is located at the top of each image. Scale bars: 500 μm.

and (4) decreased endovascular trophoblast invasion (Fig. 9). These aberrations may be related to the altered mesometrial decidua in the *Dprp*-null mouse noted above (Fig. 3). The net result is a failure in the placentation-specific adaptations to hypoxia required to ensure maintenance of pregnancy.

DISCUSSION

Decidua is a specialized uterine stromal cell modification found in species with hemochorial placentation (Aplin, 2000). It functions as a supportive structure that facilitates placentation and embryonic development and it is established that pregnancy does not proceed in its absence. In this report, we provide evidence that a secretory product of the uterine decidua is fundamental to the regulation of pregnancy-dependent adaptations to hypoxia. The decidual cell secretory product is a member of the PRL superfamily of hormones/cytokines.

The composition of the PRL superfamily is diverse and species-specific (Forsyth and Wallis, 2002; Soares, 2004). In the mouse and rat the PRL superfamily has expanded, consisting of approximately two dozen genes, whereas in other species (e.g. human and dog) the superfamily has but a single constituent (Wiemers et al., 2003; Alam et al., 2006). Why mammalian genomes evolved differently with respect to this classic hormone/cytokine is unknown. We have gained insights into the PRL superfamily through an examination of the biology of members of the expanded mouse PRL superfamily and have utilized a standard single gene mutation approach. Based on gene expression patterns, the PRL superfamily is linked to pregnancy (Soares, 2004). Previously, we demonstrated that a trophoblast cell-derived PRL family member, PLP-A (PRLPA – Mouse Genome Informatics), targets uterine NK cells and imposes only modest effects on the biology of pregnancy under standard laboratory housing conditions (Müller et al., 1999; Ain et al., 2004). In the current study, we have shown that another member of the PRL superfamily produced by uterine decidual cells, DPRP, also has subtle influences under ordinary husbandry conditions. However, both PLP-A and DPRP modulate pregnancy-dependent adaptations to hypoxia.

Wild-type pregnant mice can effectively adapt to hypoxia without fetal loss (Ho-Chen et al., 2006). Adaptations are dependent upon the timing, duration and magnitude of the hypoxic exposure. Among the pregnancy-dependent adaptations are events occurring at the maternal-fetal interface. Most notable are a compression of the mesometrial decidua and alterations in the uterine mesometrial vasculature, including its interactions with trophoblast cells. Null mutations in either the *Plp-a* gene or the *Dprp* gene interfere with adaptive responses to hypoxia and result in fetal loss. Under hypoxic conditions, the absence of PLP-A obstructs early stages of trophoblast-vascular interactions, disrupting nutrient delivery and leading to growth restriction (Ain et al., 2004). The hypoxia-exposed *Dprp*-null placenta is able to satisfactorily progress through this early interaction with the maternal environment but collapses a couple of days later, which is associated with a series of anomalies

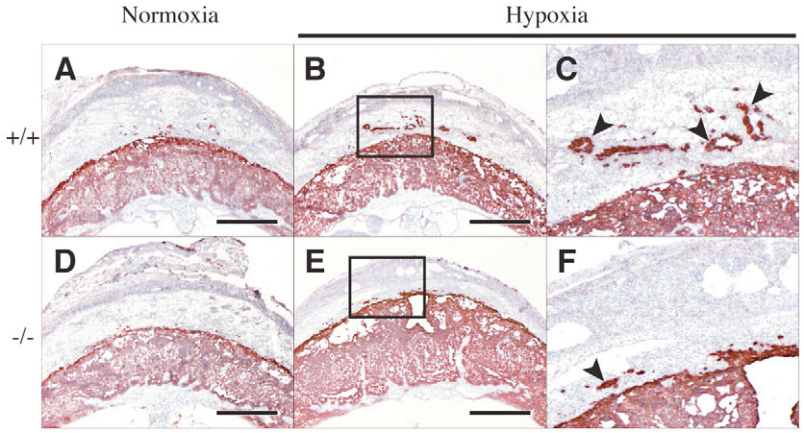

Fig. 9. Invasive trophoblast cell distribution within day 11.5 uteroplacental compartments of wild-type and *Dprp*-null mice exposed to normoxia or hypobaric hypoxia. (**A-C**) Wild-type (+/+) and (**D-F**) *Dprp*-null (–/–) mice were exposed to normoxia (A,D) or hypobaric hypoxia (B,C,E,F). Trophoblast cells were identified by cytokeratin immunostaining. C and F are high magnification images of the areas delineated by the boxes in B and E, respectively. Note the decreased endovascular trophoblast invasion (arrowheads in C,F) in the *Dprp*-null mice exposed to hypoxia. The mesometrial region of the uterus is located at the top of each image. Scale bars: 1 mm for A,B,D,E

in the uterine mesometrial compartment and placenta. The appearance of vascular lesions, enlarged mesometrial blood spaces, distorted chorioallantoic placentas, and decreased endovascular trophoblast invasion characterize the *Dprp*-null mutant response to hypoxia. The specific aberration that leads to pregnancy failure is unknown. Some insights into the *Dprp*-null phenotype may be deduced from inspection of decidual tissue adjoining the developing chorioallantoic placenta.

The orientation of the post-implantation uterus is determined by the entry site of the vasculature. The region associated with vascular entry is referred to as the mesometrial compartment, and the opposite side of the uterus is referred to as the anti-mesometrial compartment. Mesometrial and anti-mesometrial decidua differ structurally and functionally (Krehbiel, 1937; Bell, 1983; Gu and Gibori, 1995). DPRP is expressed in anti-mesometrial decidua and in a smaller population of mesometrial decidual cells situated proximal to the developing chorioallantoic placenta (Orwig et al., 1997b; Rasmussen et al., 1997) (Figs 2, 3). Decidual cell *Dprp* expression is initiated between days 5.5 and 6.5 of gestation in the mouse. In the present study, abnormalities were not observed in the organization of anti-mesometrial decidua or in its neighboring tissues from *Dprp*-null mice under normoxic or hypoxic conditions. By contrast, prominent differences were noted in the mesometrial compartments of wild-type and *Dprp*-null mice. Such observations place more significance on the mesometrial decidual cell source of DPRP. This mesometrial decidual structure may be crucial in coordinating uteroplacental adaptations to hypoxia and may provide a key to understanding the phenotype of the *Dprp*-null mouse exposed to hypoxia.

DPRP is a cytokine possessing an affinity for heparin-containing structures (Rasmussen et al., 1996; Wang et al., 2000). Evidence suggests that DPRP does not circulate but instead is deposited within the decidual extracellular matrix. Although the DPRP protein is structurally related to PRL, DPRP does not utilize the PRL-receptor signaling pathway (Rasmussen et al., 1996). The mechanism of action of DPRP is unknown but may include an autocrine/paracrine activity required for the differentiation and/or survival of the mesometrial decidua (as suggested by the present study). Alternatively, DPRP may independently modulate mesometrial vascular-trophoblast interactions. Interestingly, PRL is produced by human decidual cells, possesses an affinity for heparin (Khurana et al., 1999), and its targets are likely to be intrauterine (Jabbour and Critchley, 2001). Whether human PRL produced by decidual cells functionally overlaps with DPRP and facilitates adaptations to physiological stressors remains to be determined.

Investigation of the *Dprp*-null mouse has permitted a dissection of mechanisms controlling decidual cell adaptations to physiological stressors, and has demonstrated the effectiveness of in vivo hypoxia as a tool for elucidating intrinsic regulatory processes controlling placentation.

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