A Prolactin Family Paralog Regulates Placental Adaptations to a Physiological Stressor

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ABSTRACT

The prolactin (PRL) family of hormones and cytokines participates in the regulation of optimal reproductive performance in the mouse and rat. Members of the PRL family are expressed in the anterior pituitary, uterus, and/or placenta. In the present study, we investigated the ontogeny of PRL family 7, subfamily b, member 1 (PRL7B1; also called PRL-like protein-N, PLP-N) expression in the developing mouse placenta and established a mouse model for investigating the biological function of PRL7B1. Transcripts for Prl7b1 were first detected on Gestation Day (d) 8.5. From gestation d8.5 through d14.5, Prl7b1 was expressed in trophoblast cells residing at the interface between maternal mesometrial decidua and the developing placenta. On gestation d17.5, the predominant cellular source of Prl7b1 mRNA was migratory trophoblast cells invading into the uterine mesometrial decidua. The Prl7b1 null mutant allele was generated via replacement of the endogenous Prl7b1 coding sequence with beta-galactosidase (LacZ) reporter and neomycin cassettes. The mutant Prl7b1 allele was successfully passed through the germline. Homozygous Prl7b1 mutant mice were viable and fertile. Under standard animal housing conditions, Prl7b1 mutant mice exhibited undetectable effects on placentation and pregnancy. Hypoxia exposure during pregnancy evoked adaptations in the organization of the wild-type placenta that were not observed in Prl7b1 null placentation sites. In summary, PRL7B1 is viewed as a part of a pathway regulating placental adaptations to physiological stressors.

INTRODUCTION

The prolactin (PRL) locus in the mouse and rat encodes a family of proteins with hormone and cytokine activities contributing to the regulation of reproduction [1–3]. Members of the PRL family signaling through the PRL receptor (PRLR; defined as classical actions) have been shown to contribute to pregnancy-dependent regulation of ovarian function, mammary gland development, and islet beta cell expansion [4–6]. Most members of the PRL family do not activate their targets through the PRLR and instead possess other modes of action (defined as nonclassical actions) [1, 3]. These nonclassical PRL family members possess a broad range of pregnancy-associated cellular targets and actions, influencing vascular remodeling [7, 8], hematopoiesis [9–13], immune cell function [14, 15], and adaptations to physiological stressors [16, 17].

PRL family 7, subfamily b, member 1 (PRL7B1; also called PRL-like protein-N, PLP-N) [18] is expressed by invasive trophoblast cells (both endovascular and interstitial invasive trophoblast) at the placenta site [19, 20]. Intrauterine trophoblast cell invasion and remodeling of the uterine spiral arteries at the maternal-fetal interface are prominent features of rat, mouse, as well as human placentation [21–24]. Invasive trophoblast cells are proposed to play key roles in uterine spiral arteriole remodeling [25, 26]. Current evidence indicates that invasive trophoblast cells modulate the uterine vasculature by replacing the endothelium of the targeted uterine spiral arteries and by their production of angiogenic factors [28, 29]. Thus, Prl7b1 is situated at a key cellular site pivotal to the establishment of the hemochorial placenta; however, the physiological role of trophoblast-derived PRL7B1 is unknown. In the present study, the ontogeny of Prl7b1 expression in the developing mouse placenta was examined, a Prl7b1 null mouse model for investigating the biology of PLP-N was established, and adaptive responses to maternal hypoxia at placentation sites of wild-type and Prl7b1 null mice assessed.

MATERIALS AND METHODS

Animals and Tissue Collection

C57BL/6 mice were purchased from The Jackson Laboratory. Animals were housed in an environmentally controlled facility, with lights on from 0600 to 2000 h and were allowed free access to food and water. Timed pregnancies were generated by cohabitating male and female mice. The presence of a seminal plug in the vagina was designated as Day (d) 0.5 of gestation. Prl7b1 mutant mouse embryonic stem cells were obtained from the National Institutes of Health Knock-Out Mouse Project (KOMP) repository (www.komp.org; VG10354) [30]. The University of Kansas Medical Center Transgenic and Gene-Targeting Facility injected the mutant embryonic stem cells into albino C57BL/6 blastocysts to generate germline competent chimeras. Male chimeras were mated to C57BL/6 females to establish a germline stock of the mutant strain. Genotyping was performed using genomic DNA isolated from tail biopsies and polymerase chain reaction (PCR) with forward primers specific for the wild-type allele (5’ ctctcaagctgatcttg 3’) and mutant allele (5’ tgggccacgcttggtc 3’) and a common reverse primer (5’ cccagccagccgtaaataa 3’). PCR amplicons for the wild-type and mutant alleles were 795 and 458 bp, respectively.

Maternal hypoxia exposure was achieved by placing gestation d7.5 mice in hypoxia chambers connected to an oxygen sensor/ controller Pro-OX P110 (BioSpherix). Chambers were briefly opened each day (2–3 min) to monitor the health of the animals and replenish food and water. Tissue samples for histological analysis, including in situ hybridization and immunohistochemistry, were collected at indicated gestation days and immediately frozen in dry ice-cooled heptane and stored at −80°C until processed.

Trophoblast tissues were dissected from placentation sites from gestation d9.5 to d17.5 as previously described [31]. Briefly, trophoblast tissues were recovered from placentation sites with the aid of fine forceps and a dissecting...
microscope (10–20×). Isolated tissues represent enrichments and each contain some contaminating decidua. The dissected tissues were snap frozen in liquid nitrogen and stored at −80°C until processed for RNA extraction.

All experimentation with animals was performed in accordance with guidelines recommended by the National Institutes of Health. The University of Kansas Medical Center Animal Care and Use Committee approved the protocols for the care and use of animals.

**RNA Analysis**

RNA was extracted from tissue using TRI Reagent (Sigma-Aldrich) according to the manufacturer’s instructions. RT-PCR was performed as previously described [32]. Primer sequences used for RT-PCR, included: Prl7b1 (forward: 5′-attgccagttagctgggt 3′; reverse: 5′-ttcagtgcatcagaa 3′; amplicon: 425 bp; NM_029355) and Gapdh (forward: 5′-acctacgctgccac 3′; reverse: 5′-taaccacctgcttgta 3′; amplicon: 452 bp; NM_001289726).

**Tissue Analyses**

All histochemical staining was performed on 10 μm cryosections, which were prepared with the aid of a cryostat and stored at −80°C until use. Frozen sections were air-dried and fixed in cold phosphate-buffered saline (PBS) containing 4% paraformaldehyde except for the β-galactosidase (LacZ) histochemical staining (see below).

In situ hybridization was performed as previously described [21]. Plasmids containing cDNAs for Prl7b1, Tpbpa, and Prl2a1 were used as templates to synthesize sense and antisense digoxigenin-labeled RNA probes according to the manufacturer’s instructions (Roche Molecular Biochemicals). Prehybridization, hybridization, and detection of alkaline phosphatase-conjugated anti-digoxigenin were performed as previously reported [21].

Isolectin B4 histochemical staining was performed as previously described [17]. Endogenous peroxidase activity was quenched by incubation in methanol containing 0.3% H2O2. Sections were then incubated with PBS containing 0.1% Triton X-100 and 5 μg/ml biotinylated isoclectin B4 (B-1205; Vector Laboratories) for 30 min. Detection of isoclectin B4 binding was achieved by using ABC Kit (PK-4000; Vector Laboratories) and AEC peroxidase substrate kit (SK-4200; Vector Laboratories) according to the manufacturer’s instructions.

Immunohistochemical staining was performed as previously described [32]. Sections were incubated overnight at 4°C with antibodies against pan-cytokeratin (TROMA-I; Developmental Studies Hybridoma Repository, 1:20 dilution) to detect trophoblast cells or PECAM1 (CD31, 1:4-0311-81; eBioscience; 1:50 dilution) to detect endothelial cells. Sections for LacZ histochemical staining were air-dried and incubated in fixative solution (PBS, containing 0.1% Triton X-100 and 5 μg/ml biotinylated isoclectin B4 (B-1205; Vector Laboratories) for 30 min. Detection of isoclectin B4 binding was achieved by using ABC Kit (PK-4000; Vector Laboratories) and AEC peroxidase substrate kit (SK-4200; Vector Laboratories) according to the manufacturer’s instructions.

**Morphological Measurements**

Morphological measurements of the sizes of placental compartments were performed with National Institutes of Health Image J software as previously described [31–34]. The chorioallantoic placenta consists of the junctional zone and labyrinth zone. Definitions of the junctional zone and labyrinth zone compartments within the placenta site have been described [31]. The junctional zone consists of an area bordered by the uterine mesometrial compartments within the placentation site are described [31–34]. The chorioallantoic placenta consists of the junctional zone and labyrinth zone. The junctional zone is defined as the region of the chorioallantoic placenta vascularized by the allantosis. This compartment contains syncytiotrophoblast, cytotrophoblast, trophoblast giant cells, and fetal mesenchyme as well as its associated vasculature.

The thickness of the junctional zone was estimated from cross-sectional area measurements of isoclectin B4 histochemical-stained placenta sections. Measurements were expressed as the ratio of each zone to the total cross-sectional area of the chorioallantoic placenta (junctional + labyrinth zones) and as the ratio of the labyrinth zone cross-sectional area to the junctional zone.

**Cross-sectional area.** Chorioallantoic zone measurements were made from a histological plane at the center of each placentation site perpendicular to the flat fetal surface of the placenta. Sample sizes for the analyses were at least five placentation sites from at least five different pregnancies per treatment group.

**Statistical Analysis**

Data are presented as the mean ± standard error of the mean. Differences between two groups were assessed by Student t test. Comparisons between more than two groups were made using analysis of variance and multiple comparisons were performed using Tukey post hoc test.

**RESULTS**

**Ontogeny of Prl7b1 Expression and the Invasive Trophoblast Cell Lineage**

The invasive trophoblast cell population arises early during mouse placentation development. Prl7b1 expression can be effectively used to track the ontogeny of the invasive trophoblast cell lineage (Fig. 1A). The lineage first appears in a subpopulation of cells within the eutocplacental zone and was first evident at gestation d8.5 and continued through the course of pregnancy (Fig. 1, A and B). Prl7b1 transcripts were found in both endovascular and interstitial invasive trophoblast cell populations.

**Establishment of Prl7b1 Null Mouse**

The restricted expression of Prl7b1 in the invasive trophoblast cell lineage (Fig. 1) [19, 20] prompted an investigation of its biological role during placentation and pregnancy. Prl7b1 null mice were generated by gene-targeting strategies. The entire Prl7b1-coding region was replaced with a LacZ cassette (inserted at the ATG codon in the first exon) followed by a neomycin-resistance cassette (Fig. 2A). The mutation was successfully transmitted through the germline (Fig. 2B). Breeding of mice heterozygous for the Prl7b1 null mutation resulted in offspring genotypes that did not significantly deviate from the expected Mendelian ratio (number of litters: 29; number of pups: 201; Mendelian ratio: wild type, 45; heterozygotes, 106; Prl7b1 nulls, 50; mean litter size: 6.93). Wild-type and Prl7b1 null mutant mice exhibited similar fertility. The number of placenta and fetal and placental weights on gestation d17.5 were similar between wild-type and Prl7b1 null mice (number of placenta sites: wild type, 7.88 ± 1.0, n = 8 vs. Prl7b1 null, 8.56 ± 0.53, n = 9; fetal weights (g): wild type, 0.88 ± 0.04, n = 8 vs. Prl7b1 null, 0.79 ± 0.04, n = 9; placental weights (g): wild type, 0.095 ± 0.007, n = 8 vs. Prl7b1 null, 0.095 ± 0.002, n = 9). Furthermore, litter size was comparable for wild-type and Prl7b1 null mice at the first and second pregnancies (wild type: first pregnancy, 8.1 ± 0.9, n = 10; second pregnancy, 9.0 ± 1.2, n = 7; Prl7b1 null: first pregnancy, 8.4 ± 1.2, n = 18; second pregnancy, 8.1 ± 1.9, n = 7). Mouse placenta homozygous for the mutant allele did not express Prl7b1 (Fig. 2C) but instead exhibited LacZ activity in the invasive trophoblast cell population in a pattern similar to Prl7b1 expression in wild-type mice and confirming the integrity of the targeted mutagenesis (Fig. 3). Overall Prl7b1 null placenta sites exhibit structural and gene expression patterns (cytokeratin, Tpbpa, Prl2a1) on gestation d17.5 closely resembling wild-type placenta sites (Fig. 4). In summary, under standard laboratory housing conditions, wild-type and Prl7b1 null mice exhibited similar reproductive performance and placental structure.
Prl7b1 and Pregnancy-Dependent Adaptations to Maternal Hypoxia

The absence of a pronounced reproductive phenotype associated with the Prl7b1 null mutation was reminiscent of earlier observations of mice possessing null mutations for other members of the PRL gene family [3, 16, 17]. In these instances, null mutations at Prl4a1 and Prl8a2 loci did not significantly impact reproductive fitness under standard laboratory husbandry conditions but were each associated with compromised adaptations to maternal hypoxia. Maternal hypoxia evokes adaptive responses that alter the development of the placenta site [33, 34]. Groups of pregnant wild-type females mated with wild-type males and Prl7b1 null females mated with Prl7b1 null male were exposed to a range of oxygen tensions (9.5%, 10.5%, 11.5%, or 21% [atmospheric] O₂) from gestation d7.5 to d17.5 (Fig. 5A), which spans the time period for placental Prl7b1 expression (Fig. 1). Pregnancies from wild-type mice were more vulnerable to maternal hypoxia (9.5% O₂) and were associated with increased resorption rates, decreased fetal weights, and decreased fetal/placental weight ratios (Fig. 5, B–E). Uterine arteries were enlarged with prominent radial branches directed to each placenta site in Prl7b1 null mice exposed to low oxygen in comparison to...
similarly treated wild-type controls (Fig. 5F). In response to maternal hypoxia, wild-type placentas exhibited a striking reorganization, which included expansion of the junctional zone (Fig. 6). This is a response previously observed in pregnant rats exposed to maternal hypoxia [33, 34]. Most interestingly, these maternal hypoxia-dependent responses were not observed in placenta sites from \( \text{Prl7b1} \) null mice (Fig. 6).

Collectively, \( \text{Prl7b1} \) null pregnant mice failed to exhibit placental adaptations to maternal hypoxia. The absence of pregnancy-dependent adaptations to maternal hypoxia in \( \text{Prl7b1} \) null mice was associated with enhanced fetal survival and fetal/placental growth.

**DISCUSSION**

Expansion of the PRL family is linked to pregnancy and uteroplacental function [1, 3]. We have some knowledge of the biology of classical members of the PRL family, which influence cell signaling through activation the PRLR. Insights into the biology of nonclassical PRL members is more limited. In this report, we investigate PRL7B1, a PRL family paralog expressed in migratory trophoblast cells situated at the uterine-trophoblast interface and embedded within the differentiated uterine stroma ([19, 20] and the present study). PRL7B1 deficient mice were successfully generated using standard embryonic stem cell-mediated mouse mutagenesis. Under standard housing conditions, pregnancy outcome did not
FIG. 4. Wild-type and Prl7b1 null placentaion sites exhibit similar organization and gene expression patterns on gestation d17.5. A) Trophoblast cell distributions in wild-type (+/+) and Prl7b1 null (−/−) placentaion identified by cytokeratin immunohistochemical staining. In situ hybridization for Tpdp (B) and Prl2a1 (C) transcripts in wild-type (+/+) and Prl7b1 null (−/−) placentaion sites. Panels B and C contain low magnification (upper images) and high magnification images corresponding to the boxed areas (lower images). The red dashed lines form the boundaries of the invasive trophoblast cell infiltration into the decidual compartment. Bar = 1 mm.
appear to differ between wild-type and PRL7B1-deficient mice. Differences associated with PRL7B1 emerged when pregnant mice were exposed to hypoxia. The nature of these responses were somewhat complex. Placentas from wild-type animals exhibited a characteristic adaptation to hypoxia whereupon the junctional zone of the chorioallantoic placenta is preferentially expanded in size. This adaptive expansion was not observed in PRL7B1-deficient placentas. The outcome of the hypoxia-exposed pregnancies differed according to genotype. At gestation d17.5, uterine vascularity and fetal outcomes were superior in hypoxia-exposed PRL7B1 deficient than in hypoxia-exposed wild-type pregnancies. The results indicate that PRL7B1 contributes to the regulation of placental-associated adaptations to physiological stressors.
FIG. 6. Effects of maternal hypoxia on organization of placenta sites at gestation d17.5 in wild-type and Prl7b1 null mice. Isolectin B4 histochemical staining (A) and PECAM1 immunohistochemical staining (B) of gestation d17.5 placenta sites from wild-type and Prl7b1 null animals exposed to atmospheric oxygen (21% O₂) or maternal hypoxia (10.5% or 9.5% O₂). C) In situ hybridization for Tbpapa transcripts on gestation d17.5 placenta sites from wild-type and Prl7b1 null animals exposed to maternal hypoxia (9.5% O₂). Panel C contains low magnification (upper images) and high magnification images corresponding to the boxed areas (lower images). Bar = 1 mm. Quantitative analysis of placenta sites of wild-type and Prl7b1 null dams exposed to various oxygen tensions (wild type: 21% O₂, n = 6; 10.5% O₂, n = 5; 9.5% O₂, n = 5; Prl7b1 null: 21% O₂, n = 5; 10.5% O₂, n = 5; 9.5% O₂, n = 5). Each sample corresponds to placenta measurements from a different pregnancy. D) Junctional zone/placenta ratio. E) Labyrinth zone/placenta ratio. F) Labyrinth zone/junctional ratio. Values are presented as mean ± SEM. Letters above each mean value possessing a different identity reflect significant differences among the relevant groups (P < 0.05).
The absence of a pregnancy-related phenotype in mice with a deficiency of a PRL family member may seem perplexing but is not new. PRL4A1 and PRL8A2 are pregnancy-associated cytokines/hormones [35, 36]. Similar to the Prl7b1 null mutant mouse, genetic disruptions of Prl4A1 and Prl8A2 yield modest pregnancy-related phenotypes when examined under standard housing conditions [16, 17]. Functional redundancies are certainly possible, especially from such a large family of related proteins; however, such observations challenge the evolutionary drive for retaining these genes within the mouse genome. Instead their preservation may relate to other aspects of the reproductive success of the species, especially in less than favorable habitats. To this end, PRL4A1 and PRL8A2 have been shown to facilitate pregnancy-dependent adaptations to physiological stressors and improve pregnancy outcomes [16, 17]. Maternal hypoxia exposures led to a discrimination between wild-type and PRL7B1-deficient pregnancies but in an unexpected direction.

Maternal hypoxia exposure has been effectively used as an experimental tool to dissect pregnancy-dependent adaptations [33, 34]. Among these adaptations are structural changes in the organization of the placentation site. In the rat, maternal hypoxia exposure evokes an accelerated and increased depth of endovascular trophoblast invasion and a preferential expansion of the junctional zone relative to the labyrinth zone of the chorioallantoic placenta [33, 34]. In the mouse, structural adaptations to maternal hypoxia are restricted to expansion of the junctional zone (present study). The junctional zone is the origin of progenitors for invasive trophoblast cell development and the major placental site for maternally directed endocrine activities and energy storage [24, 37]. Hypoxia-stimulated junctional zone growth came at a cost to the development of the labyrinth zone, which is responsible for bidirectional transport of nutrients and wastes between maternal and fetal compartments. Failed pregnancies at 9.5% oxygen in wild-type females may relate to a maladaptation associated with an inappropriate balance between junctional zone and labyrinth zone development. The presence of PRL7B1 was required for hypoxia-dependent expansion of the junctional zone. PRL7B1 may possess autocrine and/or paracrine actions within the context of hypoxia-driven expansion of trophoblast cells within the junctional zone. Interestingly, another feature of the PRL7B1-positive migratory trophoblast is their accumulation in stimulating megakaryocyte growth and differentiation of glycopcyn [38, 39], which may provide a linkage to PRL7B1 actions and their involvement in adaptations to hypoxia. Relative junctional zone size has also been associated with nutrient availability. Dietary protein restriction leads to an expansion of the junctional zone [40], whereas a high fat diet is associated with attenuated junctional zone development [41]. These diet-mediated effects on junctional zone development may be secondary to the regulation of PRL7B1 expression [42]. Alternatively, PRL7B1 actions may be indirect through systemic effects on nutrient availability, which is consistent with known roles for other PRL family paralogs on maternal metabolic adaptations [43–45].

Species-specific PRL family expansions have been viewed as a strategy for improving pregnancy-dependent adaptations to environmental challenges [3]. Several PRL family members (PRL, PRL4A1, PRL8A2) improve adaptations to physiological stressors, including pregnancy outcomes [15, 16, 46–48]. Our findings with the PRL7B1-deficient mouse challenges this concept. Fetal viability at gestation d17.5 in hypoxia-exposed pregnancies was superior in females devoid of PRL7B1 in comparison to wild-type females. These observations are seemingly paradoxical and infer a level of unexpected complexity. A few possibilities are evident. First, perhaps we are not viewing the PRL7B1 phenotype along the appropriate timeline. The evolutionary drive for retention of Prl7b1 in the mouse and rat genomes may serve as a sensor of pregnancy quality and actually a successful adaptation to a stressor. Termination of pregnancies may be adaptive, preventing impending maternal demise or the production of offspring with pathologies affecting their survival. Second, it is possible that there is specificity associated with the PRL family and the nature of the physiological stressor. Maybe the Prl7b1 gene has been retained in the genome because it provides adaptive benefits to other types of physiological stressors (dietary, thermal, etc.) but not hypoxia and could represent an evolutionary prioritization of stressors. It is also conceivable that Prl7b1 is an evolutionary relic or path to a future mode of adaptation to an undetermined stressor. Third, we should also consider that the contributions of PRL7B1 to the regulation of adaptations to physiological stressors may not be essential for survival of the species. Some of these issues should clarify as we learn more about the targets and biological actions of PRL7B1.

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