

A prolactin family paralog regulates reproductive adaptations to a physiological stressor

Rupasri Ain^{*†‡}, Guoli Dai[§], Judy H. Dunmore[‡], Alan R. Godwin^{*‡}, and Michael J. Soares^{*†¶}

^{*}Institute of Maternal–Fetal Biology, [†]Division of Cancer and Developmental Biology, Department of Pathology and Laboratory Medicine, [‡]Department of Molecular and Integrative Physiology, and [§]Department of Pharmacology, University of Kansas Medical Center, Kansas City, KS 66160

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved October 7, 2004 (received for review August 22, 2004)

Successful species develop strategies to optimize their reproductive performance. This optimization likely includes the evolution of genes that specifically permit reproduction in physiologically challenging conditions. The prolactin (PRL) family gene cluster is one of 25 mouse-specific gene clusters, the majority of which are associated with reproduction. A prevailing theme characterizing the PRL family is its connection with pregnancy and mechanisms controlling viviparity. PRL-like protein A (PLP-A) is one of 26 genes located within the PRL family locus. It is a nonclassical member of the PRL family (e.g., PLP-A does not use the PRL receptor) produced by trophoblast cells of the chorioallantoic placenta and acts on uterine natural killer cells. In this report, the biology of PLP-A has been investigated by generating mice with a PLP-A null mutation. Under standardized animal husbandry conditions, PLP-A possesses modest effects on reproductive performance. However, this same gene is critical for reproduction when mice are exposed to a physiological stressor. Wild-type mice exposed to hypobaric hypoxia during gestation readily adapt and maintain their pregnancies, whereas PLP-A null mutant mice fail to adapt, resulting in pregnancy failure. PLP-A contributes to hypoxia-induced adaptations critical to hemochorial placentation and thus nutrient flow to extraembryonic and embryonic tissues. The findings provide insights into species-specific reproductive adaptations.

natural killer cell | placenta | prolactin

Reproductive adaptations to environmental challenges are a key to the success of a species (1, 2). Among mammals, few species have achieved the reproductive efficiency of mice and rats. The biological mechanisms underlying these increases in reproductive efficiency are not well understood. Sequencing of the mouse and rat genomes has provided insights that might help explain the heightened reproductive efficiencies of rodents (3, 4). The mouse and rat genomes were impacted by gene duplication and selection creating gene families not evident in other species, including the human genome. Many of these expanded gene families are associated with reproduction, as exemplified by the prolactin (PRL) gene family (3, 5).

In the mouse, the PRL family consists of at least 26 genes clustered within a 1-Mb region on chromosome 13, whereas in the human only a single member of the PRL family exists (5–7). Members of the mouse PRL gene family are expressed in tissues pivotal to the regulation of reproduction, including the anterior pituitary, uterus, and placenta (6, 7). The PRL gene family encodes for structurally related hormones/cytokines implicated in the biology of pregnancy. The best-studied member of the PRL gene family is PRL. Orthologous PRL genes are expressed in the anterior pituitary of vertebrates (8, 9). In the mouse, PRL is a key hormone regulating the development and functioning of the corpus luteum and mammary gland, actions critical to successful pregnancy and lactation (10, 11). Null mutations of either the PRL gene or the gene that encodes the PRL receptor result in female infertility (12, 13). A subset of four additional members of the mouse PRL family, referred to as placental lactogen I (α , β , and γ) and placental lactogen II and produced by the placenta during early and late pregnancy, respectively,

also activate the PRL receptor signaling pathway (5–7). The remaining 21 members of the PRL family do not appear to use the PRL receptor and are referred to as nonclassical members. Among the nonclassical members, intriguing targets and biological actions have been elucidated, including actions on cells associated with hematopoietic, vasculature, and immune systems (14–21). However, the biological significance of these nonclassical and species-specific hormones/cytokines is not known. PRL-like protein A (PLP-A) is a nonclassical member produced by trophoblast cells of the chorioallantoic placenta with actions on uterine natural killer (NK) cells (20–28). We have gained insights into the biology of PLP-A and the PRL family by generating mice with a PLP-A null mutation.

Materials and Methods

Gene Targeting. A replacement mutation was generated through the substitution of a neomycin (neo) resistance gene cassette for exons 2–5 of the mouse PLP-A gene (29). A 3.0-kb DNA fragment containing 1.5 kb of 5' flanking DNA and 1.5 kb of exon 1 and intron A of the mouse PLP-A genomic construct was subcloned upstream of the neo cassette. A 4.8-kb DNA fragment containing 3' flanking DNA located immediately downstream of exon 5 was subcloned downstream of the neo cassette and upstream of a thymidine kinase gene cassette. The accuracy of the vector was verified by restriction enzyme and DNA sequence analyses (Fig. 1). The targeting vector was introduced into R1 embryonic stem cells (ref. 30; a generous gift from Janet Rossant, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto) by electroporation. Cells were selected by exposure to G418 and ganciclovir. Southern blot analysis was used to identify clones that appropriately underwent homologous recombination with the targeting vector. Genomic DNA was isolated, digested with *Stu*I, and fractionated in 0.8% agarose gels. Southern blots were performed with a probe derived from intron A. Wild-type samples are characterized by a 14.0-kb hybridization signal; homozygous mutant samples are characterized by a 4.6-kb hybridization signal. Chimeras were generated by injection into C57BL/6 blastocysts and transferred into pseudopregnant (C57BL/6 \times CBA)F₁ females. Mice with the PLP-A mutation were backcrossed for 10 generations to the C57BL/6, 129SvJ, or CD-1 genetic background.

Western Blot Analysis. Perforin and PLP-A proteins were detected in tissue extracts by immunoblotting as described in refs. 21 and 26. Protein concentrations were determined for each sample by using the DC protein assay (Bio-Rad).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PRL, prolactin; PLP-A, PRL-like protein A; NK, natural killer.

[¶]To whom correspondence should be addressed at: Institute of Maternal–Fetal Biology, Division of Cancer and Developmental Biology, Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, KS 66160. E-mail: msoares@kumc.edu.

© 2004 by The National Academy of Sciences of the USA

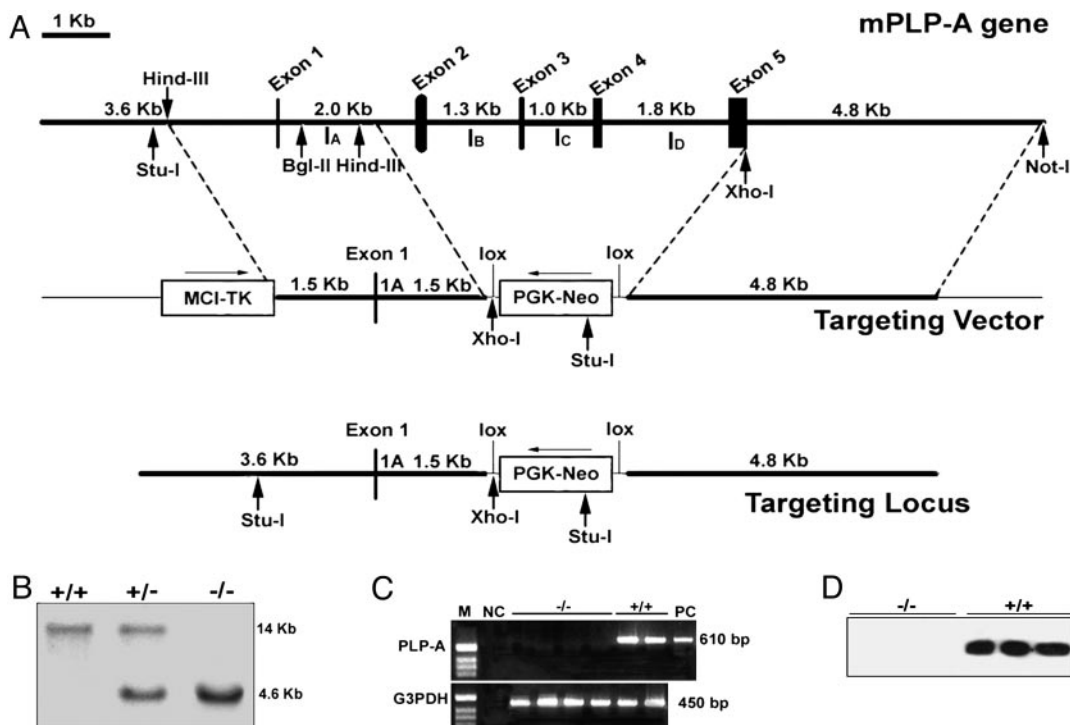


Fig. 1. Targeted disruption of the PLP-A gene in mice. (A) Schematic maps of the mouse PLP-A gene (Top), the targeting vector (Middle), and the homologous recombinant mutant allele (Bottom). (B) Southern blot analysis of tail DNA from littermates generated by heterozygous crossing. (C) RT-PCR analysis of day-11.5 placental RNA from PLP-A null mutant and wild-type mice. (D) Western blot analyses of conditioned media generated by day-11.5 placental explants cultured for 24 h in serum-free culture medium.

Immunocytochemistry. Immunocytochemical analyses were used for the purpose of determining the distribution of NK, trophoblast, and endothelial cells (31). NK cells were detected with a rabbit polyclonal anti-perforin antibody (Torrey Pines Biolabs, Houston). Trophoblast cells were monitored by using a rat monoclonal anti-mouse cytokeratin antibody (TROMA-1, Developmental Studies Hybridoma Bank, Iowa City, IA). Mesometrial endothelial cells were localized by using a rat monoclonal anti-mouse endoglin antibody (Developmental Studies Hybridoma Bank).

In Situ Hybridization. *In situ* hybridization was performed as described in ref. 31. Ten-micrometer cryosections of tissues were prepared and stored at -80°C until use. Plasmids containing cDNAs for mouse PLP-A and mouse PL-II (28, 32) were used as templates to synthesize sense and antisense digoxigenin-labeled riboprobes according to the manufacturer's instructions (Roche Molecular Biochemicals).

In Situ PLP-A Binding. PLP-A interactions with targets were evaluated by using an alkaline-phosphatase-PLP-A fusion protein (20). *In situ* alkaline-phosphatase-PLP-A binding to tissues and cells were conducted as described in ref. 20.

PRL Family Miniarray Assay. The PRL family miniarray assay is a hybridization-based tool for simultaneously monitoring expression of each member of the PRL family (32). It has been effectively used to monitor the phenotypes of the placenta and trophoblast cells. The PRL family miniarray assay was performed as described in ref. 32.

Hypobaric Hypoxia. On day 7.5 of pregnancy, female mice were placed in hypobaric chambers. Under these conditions, air is circulated at a barometric pressure of ≈ 420 torr (1 torr = 133

Pa), 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 min).

Results

Generation of a PLP-A Null Mouse. PLP-A is encoded by a five-exon-four-intron gene that possesses homology to the PRL gene structure (5, 29). PLP-A null mutant mice were generated by gene targeting strategies culminating in the replacement of a region of the PLP-A gene located between exons 2 and 5 with a neomycin resistance gene (Fig. 1 A and B). The portion of the coding sequence remaining in the mutated gene (exon 1) encodes for only the first 10 aa of the PLP-A signal peptide. Breeding of mice heterozygous for the PLP-A null mutation resulted in offspring exhibiting a typical Mendelian ratio without an apparent reproductive phenotype (Table 1). The mutation was moved to two inbred strains (C57BL/6 and 129SvJ) and the CD-1 outbred strain, after 10 generations of backcrosses.

Characterization of the PLP-A Null Mouse. Placentas from homozygous null mutant mice were devoid of PLP-A mRNA and protein, verifying the accuracy of the gene targeting and production of a null mutant (Figs. 1 C and D and 2 A–C). The absence of PLP-A and the presence of the neo resistance gene did not significantly affect expression patterns of other members of the PRL gene family (as has been seen in targeted alleles of other tandemly repeated multigene families), except for PRL-like protein F (PLP-F) (Fig. 2 A, D, and E). The impact on PLP-F was limited to day 12 of gestation and disappeared as gestation progressed. Thus, the PLP-A mutation did not appear to have a disruptive influence over other genes within the PRL gene family cluster. PLP-F has been implicated in the regulation of hematopoiesis (33), and the transient change in its expression may reflect a physiological compensation to the PLP-A defi-

Table 2. Reproductive performance in PLP-A null mutant mice

Parental genetic background	Genotype	No. of litters	No. of newborns	Litter size (mean \pm SEM)
129SvJ \times C57BL/6	+/+	6	49	8.1 \pm 0.9
	-/-	7	54	7.7 \pm 1.2
CD-1	+/+	11	145	13 \pm 2.7
	-/-	19	237	12.5 \pm 3.1
C57BL/6	+/+	12	106	8.8 \pm 1.1
	-/-	11	96	8.7 \pm 2.1
129SvJ	+/+	10	59	5.9 \pm 1.7
	-/-	14	77	5.5 \pm 1.1

conceptuses, whereas PLP-A null mutants could not sustain their pregnancies (Fig. 3 *B* and *C*). Almost all embryo-placental structures (conceptuses) of the PLP-A null mutants were undergoing resorption by day 11.5 of gestation (Fig. 3 *B* and *C*). Histological analyses of the uteroplacental structures 2 days after initiation of hypobaric hypoxia showed that the absence of PLP-A resulted in profound growth retardation of the conceptuses (Fig. 3*D*). Upon closer examination of the distribution of trophoblast (stained with TROMA-1) and endothelial (stained with endoglin antibodies) cells within the implantation sites, it became evident that trophoblast cell-vascular interactions were

impaired in the PLP-A-deficient mice exposed to hypoxia (Fig. 4 *A-F*). Between days 7.5 and 9.5 of pregnancy, there is a progression of events within the mesometrial chamber that is essential for the establishment of the hemochorial placenta (40, 41). They include degeneration of the associated uterine luminal epithelium, mesometrial-associated angiogenesis and opening of the maternal vessels into the mesometrial chamber, and expansion of the mesometrial chamber, followed by growth of the ectoplacental cone into the mesometrial chamber (40, 41). These events occur in wild-type mice and in PLP-A null mice exposed to ambient conditions. Hemochorial placentation fails in PLP-A null mice exposed to hypobaric hypoxia. The uterine epithelium lining the mesometrial chamber does degenerate, and there is a marked increase in angiogenesis within the mesometrial uterine compartment; however, trophoblast cells associated with the ectoplacental cone do not grow into the mesometrial chamber (compare *E* and *F* in Fig. 4; summarized in *G*). The net result is a failure of adequate nutrient flow to support extraembryonic and embryonic development.

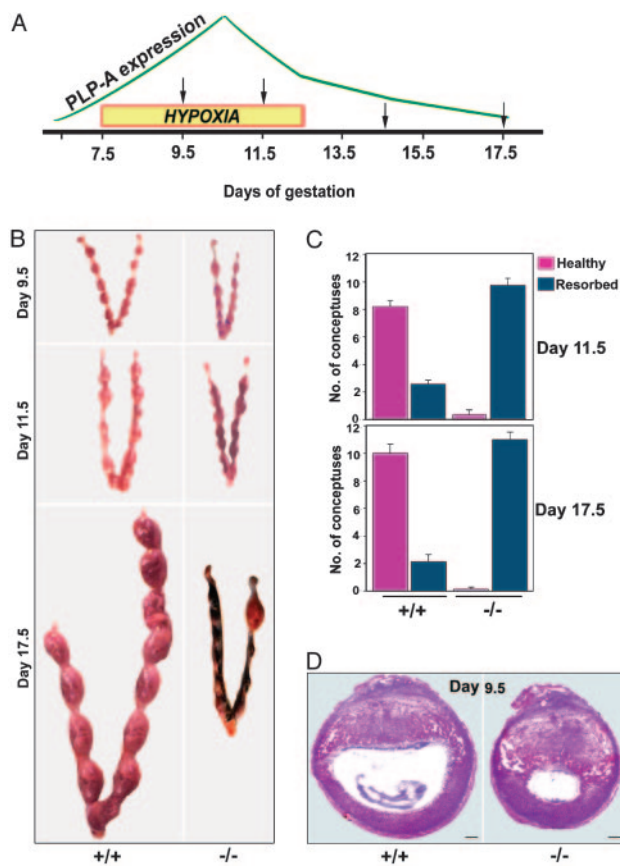


Fig. 3. Pregnancies in PLP-A null mice are vulnerable to maternal hypoxia. (A) Schematic representation of placental PLP-A expression patterns (18–20), maternal hypoxia exposure, and gestation days for tissue harvesting. (B) Pregnant uteri of wild-type and PLP-A null mutant mice exposed to hypoxia. (C) Quantitation of pregnancy outcomes in wild-type and PLP-A null mutant mice exposed to hypoxia. (D) Hematoxylin/eosin stained sections of implantation sites in wild-type and PLP-A null mutant mice after 48 h of hypoxia exposure. (Scale bars: 250 μ m.)

PLP-A Influences Mesometrial IFN- γ Expression. The mechanisms underlying the increased sensitivity to hypoxia during pregnancy in PLP-A null mice are not known. Uterine NK cells may be involved. They associate with the uterine mesometrial vasculature, and their function is affected by PLP-A (20, 21). Uterine mesometrial NK cells contribute to the uteroplacental cytokine milieu through their production of IFN- γ (42). PLP-A is a negative modulator of uterine mesometrial NK cell production of IFN- γ . PLP-A inhibits NK cell production of IFN- γ *in vitro* (21), and uterine mesometrial IFN- γ concentrations are significantly increased in PLP-A null mutant conceptuses (Fig. 5). IFN- γ is known to inhibit trophoblast growth (31) and may be a contributing factor to the trophoblast growth restriction seen in the pregnant PLP-A null mice exposed to hypoxia; however, dysregulated IFN- γ is not the complete explanation. Uterine mesometrial IFN- γ levels are also elevated in the pregnant PLP-A null mouse housed under ambient conditions, yet their conceptuses do not exhibit any evidence of growth arrest. Any involvement of NK cells or IFN- γ in the adaptations to maternal hypoxia will need to be directly evaluated.

Discussion

Investigation of the phenotype of the PLP-A null mouse has generated unexpected insights into mechanisms underlying species-specific reproductive adaptations. PLP-A, a placental hormone and member of the mouse PRL family, is dispensable when mice are maintained under standard animal husbandry practices. However, it is evident that the selective pressures driving the evolution of the mouse PRL family or, more specifically, the PLP-A gene did not occur in the laboratory with the availability of a reliable food supply and modern veterinary hygienic practices. Accordingly, we reasoned that the biological importance of PLP-A might become apparent when the mice

Additional experimentation will be required to determine the precise involvement of uterine NK cells and IFN- γ in the adaptive responses mediated by PLP-A.

Species-specific gene-family expansions appear to represent a conserved mechanism contributing to the regulation of reproductive adaptations. The mouse and rat have used the ancestral PRL gene as a template to create an assortment of pregnancy-specific modulators. Other species-specific gene families linked to pregnancy exist in the mouse and rat (3, 4), as well as in other mammals (47, 48). The choice of template for the species-specific gene-family expansions is sometimes conserved across divergent species and other times restricted to only more closely related species. Each pregnancy-associated gene-family expansion likely represents a strategy for improving reproductive success. Although PRL-family ligands are not well conserved between mice and humans (6, 7), the selection of targets for the murine PRL family ligands is intriguing. It is logical to assume that modulating the functioning of hematopoietic, vascular, and immune systems during pregnancy represents an effective strategy for ensuring the safe flow of nutrients from mother to fetus (14–21).

1. Bock, W. J. (2003) *Zool. Sci.* **20**, 279–289.
2. Feder, M. E. & Mitchell-Olds, T. (2003) *Nat. Rev. Genet.* **4**, 649–655.
3. Mouse Genome Sequencing Consortium (2002) *Nature* **420**, 520–562.
4. Rat Genome Sequencing Project Consortium (2004) *Nature* **428**, 493–521.
5. Wiemers, D. O., Shao, L.-J., Ain, R., Dai, G. & Soares, M. J. (2003) *Endocrinology* **144**, 313–325.
6. Soares, M. J. & Linzer, D. I. H. (2001) in *Prolactin*, ed. Horseman, N. D. (Kluwer, Boston), pp. 139–167.
7. Soares, M. J. (2004) *Reprod. Biol. Endocrinol.* **2**, 51, www.rbej.com/content/2/1/51.
8. Bern, H. A. & Nicoll, C. S. (1968) *Recent Prog. Horm. Res.* **24**, 681–720.
9. Forsyth, I. A. & Wallis, M. (2002) *J. Mammary Gland Biol. Neoplasia* **7**, 291–312.
10. Goffin, V., Binart, N., Touraine, P. & Kelly, P. A. (2002) *Annu. Rev. Physiol.* **64**, 47–67.
11. Bole-Feysot, C., Goffin, V., Edery, M., Binart, N. & Kelly, P. A. (1998) *Endocrine Rev.* **19**, 225–268.
12. Horseman, N. D., Zhao, W., Montecino-Rodriguez, E., Tanaka, M., Nakashima, K., Engle, S. J., Smith, F., Markoff, E. & Dorshkind, K. (1997) *EMBO J.* **16**, 6926–6935.
13. Ormandy, C. J., Camus, A., Barra, J., Damotte, D., Lucas, B., Buteau, H., Edery, M., Brousse, N., Babinet, C., Binart, N. & Kelly, P. A. (1997) *Genes Dev.* **11**, 167–178.
14. Jackson, D., Volpert, O. V., Bouck, N. & Linzer, D. I. H. (1994) *Science* **266**, 1581–1584.
15. Bengtson, N. W. & Linzer, D. I. H. (2000) *Mol. Endocrinol.* **14**, 1934–1943.
16. Lin, J. & Linzer, D. I. H. (1999) *J. Biol. Chem.* **274**, 21485–21489.
17. Bhattacharyya, S., Lin, J. & Linzer, D. I. (2002) *Mol. Endocrinol.* **16**, 1386–1393.
18. Bittorf, T., Jaster, R., Soares, M. J., Seiler, J., Brock, J., Friese, K. & Müller, H. (2000) *J. Mol. Endocrinol.* **25**, 253–262.
19. Wang, D., Ishimura, R., Walla, D. S., Müller, H., Dai, G., Hunt, J. S., Lee, N. A., Lee, J. J. & Soares, M. J. (2000) *J. Endocrinol.* **167**, 15–29.
20. Müller, H., Liu, B., Croy, B. A., Head, J. R., Hunt, J. S., Dai, G. & Soares, M. J. (1999) *Endocrinology* **140**, 2711–2720.
21. Ain, R., Tash, J. S. & Soares, M. J. (2003) *Mol. Cell. Endocrinol.* **204**, 65–74.
22. Duckworth, M. L., Peden, L. M. & Friesen, H. G. (1986) *J. Biol. Chem.* **261**, 10879–10884.
23. Deb, S., Youngblood, T., Rawitch, A. & Soares, M. J. (1989) *J. Biol. Chem.* **264**, 14348–14353.
24. Campbell, W. J., Deb, S., Kwok, S. C., Joslin, J. A. & Soares, M. J. (1989) *Endocrinology* **125**, 1565–1574.
25. Deb, S. & Soares, M. J. (1990) *Mol. Cell. Endocrinol.* **74**, 163–172.
26. Deb, S., Hamlin, G. P., Kwok, S. C. M. & Soares, M. J. (1993) *J. Biol. Chem.* **268**, 3298–3305.
27. Lin, J., Poole, J. & Linzer, D. I. H. (1997) *Endocrinology* **138**, 5541–5549.
28. Müller, H., Ishimura, R., Orwig, K. E., Liu, B. & Soares, M. J. (1998) *Biol. Reprod.* **58**, 45–51.
29. Dai, G., Chapman, B. M., Wang, D., White, R. A., Preuett, B. & Soares, M. J. (1999) *Mammal. Genome* **10**, 78–80.
30. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W. & Roder, J. C. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8424–8428.
31. Ain, R., Canham, L. N. & Soares, M. J. (2003) *Dev. Biol.* **260**, 176–190.
32. Dai, G., Lu, L., Tang, S., Peal, M. J. & Soares, M. J. (2002) *Reproduction* **124**, 755–765.
33. Zhou, B., Lum, H. E., Lin, J. & Linzer, D. I. H. (2002) *Endocrinology* **143**, 4281–4286.
34. Guimond, M. J., Luross, J. A., Wang, B., Terhorst, C., Danial, S. & Croy, B. A. (1997) *Biol. Reprod.* **56**, 169–179.
35. Ashkar, A. A. & Croy, B. A. (2001) *Semin. Immunol.* **13**, 235–241.
36. Czyzyk-Krzeska, M. F. (1997) *Respir. Physiol.* **110**, 99–111.
37. Bruick, R. K. & McKnight, S. L. (2001) *Genes Dev.* **15**, 2497–2502.
38. Semenza, G. L. (2003) *Annu. Rev. Med.* **54**, 17–28.
39. Zamudio, S. (2003) *High Alt. Med. Biol.* **4**, 171–191.
40. Welsh, A. O. & Enders, A. C. (1991) *Am. J. Anat.* **192**, 347–365.
41. Enders, A. C. & Welsh, A. O. (1993) *J. Exp. Zool.* **266**, 578–587.
42. Ashkar, A. A. & Croy, B. A. (1999) *Biol. Reprod.* **61**, 493–502.
43. Kaplan, S. L. & Grumbach, M. M. (1981) in *Fetal Endocrinology*, eds. Novy, M. J. & Resko, J. A. (Academic, New York), pp. 127–139.
44. Dorshkind, K. & Horseman, N. D. (2001) *BioEssays* **23**, 288–294.
45. Dugan, A. L., Thellin, O., Buckley, D. J., Buckley, A. R., Ogle, C. K. & Horseman, N. D. (2002) *Endocrinology* **143**, 4147–4151.
46. Dugan, A. L., Schwemberger, S., Babcock, G. F., Buckley, D., Buckley, A. R., Ogle, C. K. & Horseman, N. D. (2004) *Shock* **21**, 151–159.
47. Xie, S., Green, J., Bixby, J. B., Szafranska, B., DeMartini, J. C., Hecht, S. & Roberts, R. M. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 12809–12816.
48. Roberts, R. M., Ezashi, T., Rosenfeld, C. S., Ealy, A. D. & Kubisch, H. M. (2003) *Reprod. Suppl.* **61**, 239–251.

A hierarchy of genes critical to reproduction can be constructed. Key conserved genes regulating the hypothalamic–anterior pituitary–gonadal axis and the uterus (e.g., gonadotropin-releasing hormone, luteinizing hormone, progesterone receptor, etc.) are fundamental regulators of reproduction across a broad spectrum of vertebrates. PLP-A and, likely, other nonorthologous members of the PRL family improve reproductive efficiency and promote adaptive responses to physiological stressors, but they are not intrinsic regulators of reproduction. Such genes have been retained in the mouse and rat genomes because they provide selective advantages. They allow species to reproduce in a range of habitats during an assortment of environmental challenges.

We thank Drs. Norberto C. Gonzalez, John G. Wood, and William E. Truog for helpful discussions on the biology of hypoxia; Dustin O. Wiemers, Lu Lu, and My-Linh Trinh for technical assistance; and Kathryn Vasicek for animal care. This work was supported by grants from the National Institutes of Health, the Hall Family Foundation, the Philip Astrowe Trust, and the American Heart Association.