

# **PROLACTIN**

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# Chapter 8

## Rodent Prolactin Family and Pregnancy

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**INTRODUCTION** Rodents have exploited the ancestral PRL gene to evolve a large number of closely related genes encoding PRL-like proteins. These PRL-related genes are all expressed at the implantation site, either in the trophoblasts of the placenta or in the decidual cells of the uterus. Some of these hormones have bioactivities indistinguishable from PRL, whereas most others have distinct molecular and cellular targets, and as a consequence distinct activities. This chapter will review what is currently known about this family of hormones.

**DISCOVERY OF THE RODENT PROLACTIN FAMILY** The rodent placenta has long been recognized as an endocrine organ that produces hormones related to PRL. Both luteotropic and mammatropic activities, indistinguishable from the effects of PRL, were reported for rat placental extracts well before the responsible factors were characterized (1, 2). The assignment of these activities in extracts to specific protein hormones was made possible by the development of a radioreceptor assay for ligand binding to the PRL receptor (PRL-R; 3). Surprisingly, this assay revealed two distinct peaks of activity from the rat and mouse placenta during gestation, one peak at mid-pregnancy and a second peak in late-pregnancy (4, 5). That these two activities were not identical was demonstrated by the development of specific radioimmunoassays (6, 7), and later by the isolation of cDNA clones for the two hormone mRNAs (8-11). Based on the source of these proteins and their ability to stimulate milk production, they were named placental lactogen I (PL-I) and II (PL-II). As expected from bioassays and receptor binding assays, PL-I and PL-II are closely related to PRL, in the mouse sharing 32 and 37 percent amino acid sequence identity with PRL, respectively.

Between the time of discovery of PL-I and PL-II as proteins secreted by the placenta, and the cloning of the PL-I and PL-II cDNAs, two other PRL-related proteins were found. A search in mouse fibroblasts for factors that might be involved in cell cycle regulation led to the isolation of several serum-inducible mRNAs (12). The analysis of one of these cDNA clones represented one of the first examples of successful database comparison and placement of a novel gene within a known gene family (13). William

Pearson, then a postdoctoral fellow at The Johns Hopkins University School of Medicine, had just written an initial version of the sequence similarity analysis program known as FAST A, and was seeking a sequenced cDNA clone encoding an unknown protein. A partial cDNA clone, isolated and sequenced in an adjacent laboratory, was available to test FAST A. What was also needed was a sequence library, but no nucleotide databases had yet been compiled in 1983. Instead, Pearson had access to a minimal protein sequence database that was maintained on a computer at Stanford University. That database included sequences obtained by direct protein sequencing, and was therefore limited to proteins such as insulin, cytochrome C, and PRL and GH. The inclusion of PRL and GH in the database resulted from the pioneering efforts of C.H. Li at the University of California. Despite the low probability of success of analyzing a partial cDNA clone with a new algorithm by comparison to an extremely minimal database, a significant sequence similarity was found between the test sequence and PRL. As a result of the sequence similarity to prolactin, and because of the *proliferation*-dependent pattern of expression in mouse fibroblasts, the encoded protein was named *proliferin* and abbreviated PLF (14). Several years later, it became recognized that PLF and a "mitogen-regulated protein" or MRP found previously in cultured mouse fibroblasts were the same (15).

Taken together, the earlier characterization of PL-I and PL-II proteins as close relatives of PRL and the identification of a novel cDNA clone encoding a protein also related to PRL suggested that PLF might actually correspond to PL-I or PL-II. Consistent with this hypothesis, a PLF-hybridizing mRNA was detected specifically in the placenta (16) and with a time course similar to that of PL-I synthesis. To confirm that the placental mRNA hybridizing to the PLF cDNA was in fact the same as that isolated from mouse fibroblasts, the placental mRNAs were also isolated as clones. This analysis revealed that PLF is expressed in the placenta, but also led to two unexpected findings. First, the PLF cDNA clones were found to be nearly but not completely identical; subsequent analysis has shown that several PLF genes are present in the mouse genome and are transcribed into apparently functional mRNAs encoding slightly different proteins (17, 18). Second, a weakly hybridizing cDNA clone was also isolated and was found to encode yet another protein similar to PRL but distinct from PLF; this protein was therefore named PLF-related protein or PRP (19). PRP was also found to be expressed specifically in the placenta, with a time course similar to that of PL-II.

The hypothesis that the PLF and PRP cDNA clones correspond to the mouse protein hormones PL-I and PL-II was attractive, but no PRL-like bioactivity was detected for PLF or PRP (20, 21). Indeed, subsequent cloning of the mouse PL-I and PL-II mRNA~ (9, 10) demonstrated that these hormones are distinct from PLF and PRP. Thus, it seemed that these two cDNA clones might define a new class of PRL-related hormones that had escaped prior detection in assays for proteins with receptor binding properties and actions similar to those of PRL or GH. What was completely unexpected was the vast numbers of these "orphan" PRL-related hormones that would be discovered in the mouse and rat in the following years. These hormones, which now number approximately two dozen, were discovered during the characterization of the available members of the PRL family at protein, cDNA, and genomic levels, by differential display and screening of placental RNAs, and, most recently, by computer searches of expressed-sequence tag (EST) databases. Upon completion of the mouse genome sequencing project, the actual number of distinct genes in the family and their relative chromosomal positions will be defined, but variations in post-translational modification of the hormone products (and potentially in the primary translation products as a result

of alternative mRNA splicing) are expected to add even more complexity to the spectrum of PRL-related placental hormone structures and functions.

### **NOMENCLATURE**

The PRL family consists of a group of structurally-related proteins. Nomenclature for members of the PRL family reflects biological activities (PLs), structural relationships with PRL (PRL like proteins, PRL related proteins), or association with proliferation (PLF). Shared functional characteristics with pituitary PRL are not a prerequisite for inclusion in the PRL family. Over a dozen different laboratories have participated in the discovery and naming of members of the rodent PRL family. Accordingly, it is not surprising that terminology for some PRL family members is confusing. In Table 8-1, we have attempted to clarify ambiguities associated with the names for members of the rodent PRL family that currently exist in the literature. Once we have a better appreciation for the size of the PRL family and the interrelationships of its members, it will be advantageous and warranted to devise a unified and more appropriate nomenclature.

**STRUCTURAL CHARACTERISTICS** Hormones in the PRL/GH family share several features. All are synthesized as preproteins with co-translational cleavage of an amino-terminal, hydrophobic secretion signal sequence of approximately 30 residues. In each case, the remainder of the primary translation product is then secreted as an intact polypeptide chain of approximately 200 amino acids in length. Many (but not all) of these hormones include consensus sites for N-linked glycosylation. Although variations in glycoforms has not been shown to give rise to significant differences in the bioactivities of these placental hormones, ovine, porcine, and human PRL glycoforms have been reported to differ in activity (50-54). No post-translational modifications of these proteins other than glycosylation have been reported, although it remains possible that other modifications occur such as cleavage or phosphorylation to generate hormones with distinct bioactivities, as has been found for PRL (55, 56). The primary amino acid sequence also includes several highly conserved cysteine residues, but variations have been found in the pattern of cysteines and in the formation of disulfide linkages.

The crystal structure of GH reveals it to be a four helix bundle protein (57). The primary amino acid sequences of the placental hormones are compatible with four alpha-helical regions, and the recent crystal structure for ovine PL complexed to the PRL receptor is consistent with this prediction (58). In the two independent regions of the hormone known to be responsible for contact of PRL and GH with their receptors in a 1 hormone: 2 receptor complex (58-60), critical residues are found to be common among PRL, PL-I, and PL-II, and absent in PLF and PRP (61). It is not known, though, if variations of amino acid sequence in these regions confer distinct receptor specificities for PLF and PRP; or if receptor interaction occurs at unrelated sites in these hormones. While the former prediction would be most consistent with a common structural basis for binding of cytokines to cytokine receptors, the ability of PLF to interact (at least in part) through its carbohydrate moiety with the insulin-like growth factor II/mannose 6-phosphate receptor (21) suggests that the topology of the PRL/PRL-R and GH/GH-R interactions may not translate to the complexes of all of the other related hormones and their corresponding receptors.

The alpha-helical and putative receptor binding domains do not equate to individual gene exons, and in fact the receptor binding domains for GH actually correspond

Table 8-1. Rat and Mouse Prolactin Family

Member	Other names	Species	GenBank Accession	Refs
<b>Classical (4)</b>				
PRL*		Rat, mouse	V01249, X02892	22, 23
PL-I	Midpregnancy lactogen, PL-Im	Rat, mouse	M55269, D21103, M35662	10, 11, 24, 25
PL-IV		Rat	M55269, U32679	26-28
PL-II	PL	Rat, mouse	M13749, M14647	8, 9
<b>Nonclassical (17)</b>				
<i>PLF subfamily</i>				
PLF	MRP	Mouse	K02245, X16009	14, 29, 30
PRP	PLF-RP	Rat, mouse	AF139809, X02594, AF226609	19, 31, 32
PLP-A		Rat, mouse	M13750, AF011383, AF015562	33-35
PLP-B		Rat, mouse	M31155, AF011384, AF015563	34-36
<i>PLP-C subfamily</i>				
PLP-C		Rat	M76537	37
PLP-Cv		Rat	U93351	38
PLP-D		Rat	AB000107	39
PLP-H	PLP-E	Rat	AB009889	40
D/tPRP	DPRP		L06441, AF015729, AF011385	34, 41, 42
PLP-Cv		Mouse	AF090140	43
<i>PLP-E/F subfamily</i>				
PLP-E	PLP-G	Mouse	AF011381, AF020525	44, 45
PLP-F		Rat, mouse	AF139808, AF011382, AF02054	31, 44, 45
PLP-I		Rat	AB019791	46
PLP-J	PLP-I, Decidualin	Rat, mouse	AB019945, AB019119, AF150741, AF234638, AB019118, AF150740, AF234637	46-49
PLP-K		Rat, mouse	AB022882, AF234635	46, 49
PLP-L		Rat, mouse	AB022883, AF226607, AF226611	45, 32
PLP-M		Rat, mouse	AF226608, AF226610, AF234636	32, 49

\*Abbreviations: PRL, prolactin; PL, placental lactogen; PLP, prolactin-like protein; d/tPRP or DPRP, decidual/trophoblast prolactin-related protein; PLF, proliferin; MRP, mitogen regulated protein PRP nr PLF-RP, proliferin related protein.

to three dimensional structures that form from discontinuous stretches of the polypeptide chain (60). Nevertheless, the number and size of the exons, and the locations of interruptions in the genes by introns, are highly conserved with one major exception. While most of the genes encoding hormones in the family consist of five exons, genes for PRP and the PLP-C subfamily (see Table 8-1) include an extra, small third exon that encodes a segment of approximately 12 amino acids (38,43,62,63). All of the genes for the placental PRL family of hormones in mice have been mapped to the same region of chromosome 13 that contains the PRL gene (see the Mouse Genome Database at [www.informaticsjax.org](http://www.informaticsjax.org) for the most current information). The proximity of these genes to the PRL gene, and the closer relationship between the sequences of the placental hormone genes and the PRL gene than the GH gene, argue that an ancestral PRL gene gave rise to multiple gene copies and that these gene copies subsequently diverged to encode distinct protein products.

## EXPRESSION

During pregnancy the expression of the PRL family is precisely orchestrated in spatial and temporal patterns (see Tables 8-2 and 8-3). There are three main lineages of cells involved in the biosynthesis of members of the PRL family: *i*) lactotropes of the anterior pituitary, *ii*) decidual cells of the uterus, and *iii*) trophoblast cells of the placenta. The cell biology of the lactotrope is presented elsewhere in this volume. Below we present an overview of the organization of the uteroplacental compartment, describe cellular and temporal expression patterns, and discuss possible mechanisms controlling expression of the PRL family. We will focus on expression characteristics relevant to the biology of each hormone as a regulator or potential regulator of the gestational state. Factors controlling the expression of members of the PRL family are tightly coupled to

**Table 8-2. Tissue Distribution**

<b>Tissue Distribution</b>	<b>PRL Family Member</b>	<b>Refs</b>
<i>Maternal compartment</i>		
Anterior pituitary	PRL	64
Decidual cell	PLP-B, d/tPRP, PLP-J, PRL	34, 35, 41, 42, 48, 49, 65-68
<i>Extraembryonic compartment</i>		
Trophoblast giant cell (TGC)	PL-I, PL-II, PLF, PLP-E	44, 69-75
Spongiotrophoblast cells	PLP-B, PLP-F (mouse)	66, 72, 34, 35
TGC & spongiotrophoblast cells	PL-Iv, PLP-A, PLP-C, PLP-Cv, d/tPRP, PLP-D, PLP-H, PLP-Cv, PRP (mouse), PLP-F (rat), PLP-K (mouse), PLP-M	26, 31, 38-40, 43, 49, 67, 71, 72, 75-77
Labyrinthine trophoblast	PL-II, PRP (rat), PLP-K	26, 31, 49
Endovascular trophoblast	PRP, PLP-L, PLP-M	32
<i>Fetal compartment</i>		
Anterior pituitary	PRL	78-81

Table 8-3. Temporal Patterns of Expression

Member	Temporal profile				Refs
	Pre-implant	Post-implant	Mid gestation	Late gestation	
<b>Classical members</b>					
PRL	X	X		X	64, 78, 80
PL-I		X	X		10, 73, 82, 83
PL-IV			X	X	26, 27, 84
PL-II			X	X	8, 9, 71, 73
<b>Nonclassical members</b>					
<i>PLF subfamily</i>					
PLF		X	X		16, 70
PRP			X	X	19, 76
<i>PLP-E/F subfamily</i>					
PLP-E		X	X		44, 45
PLP-F			X	X	31, 44, 45
<i>Decidual family</i>					
PLP-B		X	X		36, 65, 66
PLP-J		X	X		48, 49
D/TPRP		X	X	X	67, 85
PLP-A			X	X	33, 71
PLP-K			X	X	49
PLP-M			X	X	49
<i>PLP-C subfamily</i>					
PLP-C				X	37, 77
PLP-Cv				X	38
PLP-D				X	39
PLP-H				X	40
PLP-C $\alpha$				X	43

\*X denotes expression of the PRL family member as monitored by immunocytochemistry, in situ hybridization, Northern blotting, Western blotting, and/ or immunoassays.

the differentiation of the cells responsible for their production. Thus, insight into the control of PRL family gene expression potentially provides clues into the regulation of lactotrope, decidual cell, and/or trophoblast cell differentiation. Conversely, information regarding the differentiation of these cell types similarly enhances our understanding of the control of PRL family gene expression.

#### ORGANIZATION OF THE UTEROPLACENTAL COMPARTMENT

In order to proceed with a discussion of expression patterns for members of the PRL family it is necessary to introduce terminology pertinent to the morphology of the

mouse and rat uteroplacental unit. Our account of these organizational features is based on earlier reports (86-91). Features of the organization of the uteroplacental compartment are depicted in Figure 8-1.

In the rodent, trophoblast and decidual lineages characterize pregnancy. The trophoblast lineage first arises after the morula stage of embryogenesis. Cells on the outer layer of the morula, *trophectoderm*, become distinguishable from those in the inner core, *inner cell mass* (ICM). Trophectoderm cells are the progenitors of trophoblast cells of the placenta, while the ICM will go on to differentiate into embryonic and other extraembryonic tissues. At the stage of blastocyst implantation, *mural* trophectoderm cells, located farthest from the

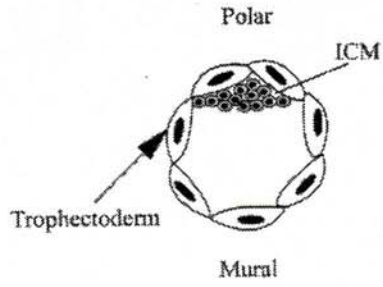
ICM, initially adhere to *antimesometrial* (farthest from the incoming uterine blood supply) uterine epithelial cells and then invade into the uterine stroma initiating a transformation of the stromal cells termed *decidualization*.

Decidualization is dependent upon the hormonal milieu (estrogen and especially progesterone) and can be induced by the blastocyst resulting in the formation of *decidual tissue*. Trophoblast lineages go on to contribute to the formation of two placental structures in the rodent: *i*) the *choriovitelline* placenta and *ii*) the *chorioallantoic* placenta. The choriovitelline placenta develops first, and is comprised of mural trophoblast cells adherent to a prominent basement membrane, *Reichert's membrane* (secreted by *parietal endoderm* cells) and is associated with decidual cells, termed *decidua capsularis*. The choriovitelline placenta eventually degenerates and disappears shortly after midpregnancy. The chorioallantoic placenta develops before the demise of the choriovitelline placenta and is located in the *mesometrial* region of the uterus (closest to the incoming blood supply). The chorioallantoic placenta is comprised of *polar* trophoblast cells which initially organize into a structure referred to as the *ectoplacental cone* and then as gestation progresses contribute to the formation of two structurally and functionally distinct regions: *i*) *junctional zone* and *ii*) *labyrinth zone*. *Basal zone* is a term sometimes used synonymously with junctional zone. The junctional zone is located adjacent to the *decidua basalis* and contains trophoblast cells and maternal vascular channels but is devoid of fetal vessels, while the labyrinth zone is located adjacent to the developing embryo and contains trophoblast cells, maternal vascular channels, and fetal vessels. The size and complexity of these placental zones change as gestation proceeds. A number of differentiated trophoblast cells can be identified in the junctional zone (*trophoblast giant cells*, *spongiotrophoblast cells*, *glycogen cells*) and in the labyrinth zone (*labyrinthine trophoblast stem cells*, *labyrinthine trophoblast giant cells*, *syncytiotrophoblast cells*). An additional population of cells derived from the trophoblast lineage and associated with the chorioallantoic placenta is referred to as *endovascular trophoblast*. This lineage of trophoblast cells is invasive and supplants the endothelium of uterine blood vessels.

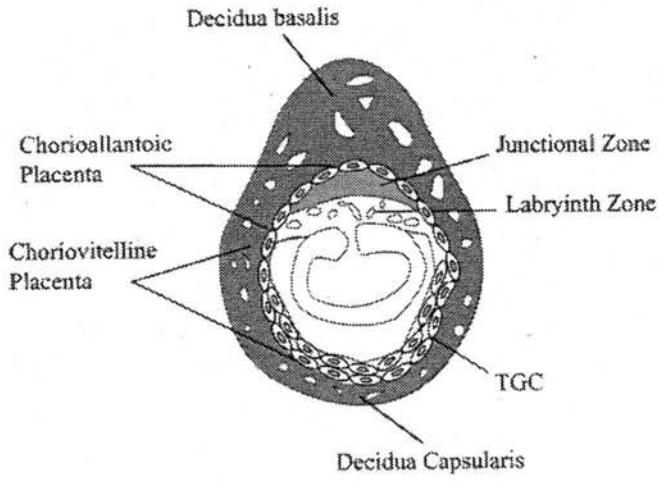
### *PATTERNS OF EXPRESSION*

Individual PRL family members can be expressed by a single cell type or by multiple cell types. Expression of the same PRL family member in more than one cell type has important implications. Cellular source directly impacts posttranslational processing and thus potentially the distribution and activity of the ligand. Sites of ligand biosynthesis also affect the ligand's access to potential cellular targets.

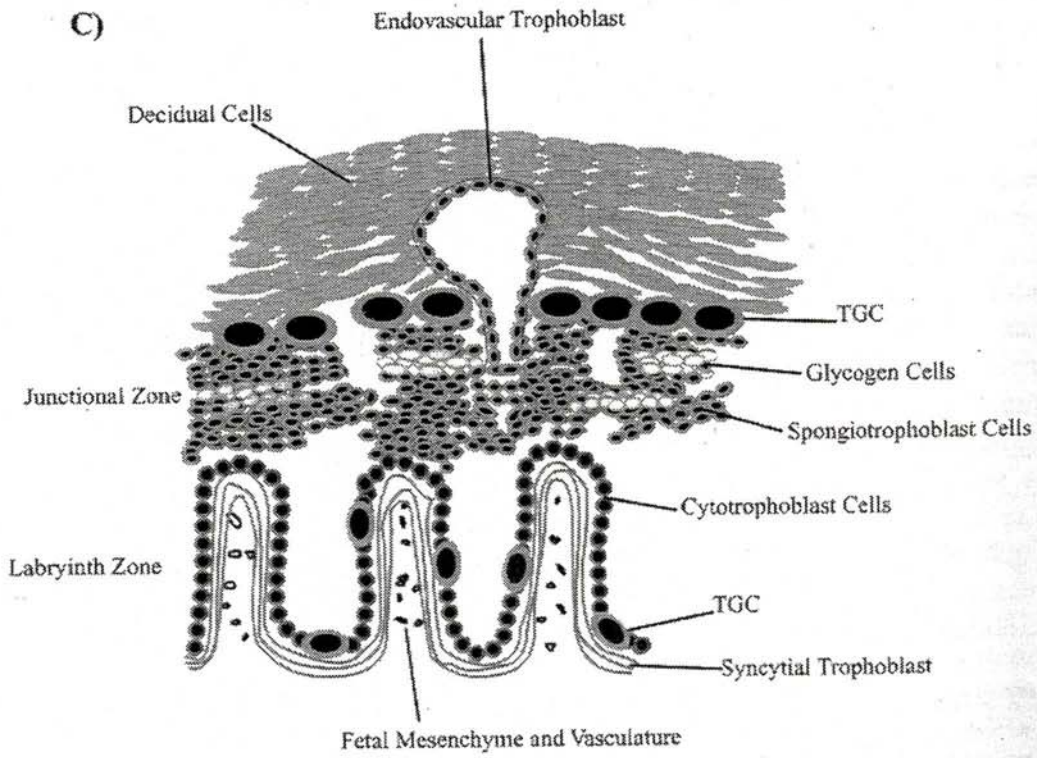
A)



B)



C)



*DECIDUAL CELL* Decidual cells arise from uterine stromal cells under the control of progesterone and contribute to the production of four members of the PRL family: PRL-like protein-B (PLP-B; 34, 35, 65, 66), decidual/trophoblast PRL-related protein (d/tPRP; 34,41,42,67, 85), PLP-J (47-49), and PRL (68). The site of expression for each of these members is primarily confined to the antimesometrial deciduum and is independent of the presence of embryonic or extraembryonic structures. The time course of expression follows the life span of decidua. While decidual cells appear to be the sole source of PLP-J; PLP-B and d/tPRP are also produced by trophoblast cells of the chorioallantoic placenta and of course, the primary source of PRL is the anterior pituitary.

A few insights are available regarding the regulation of a couple of decidual PRL family genes. Decidual cell expression of PRL is stimulated by progesterone and cyclic AMP and inhibited by PRL (68). Since progesterone and cyclic AMP promote decidual cell differentiation, they will also likely impact the biosynthesis of PLP-B, d/tPRP, and PLP-J (92). These decidual cell regulators also stimulate PRL gene expression in human decidual cells (92). The gene for rat d/tPRP has been cloned and its 5' flanking DNA has been partially characterized (62, 93). Putative activator protein-1 (AP-1) and Ets elements within the 93 bp proximal promoter participate in the transcriptional control of the d/tPRP gene in decidual cells (93). Decidual proteins binding to these regulatory sites have not been identified, nor have the upstream pathways controlling d/tPRP gene expression. We would anticipate that at least some transcriptional controls will be shared among each PRL family gene expressed in decidual cells.

*TROPHOBLAST GIANT CELL* Trophoblast giant cells are the most versatile of the cellular sources, possessing the capacity to express most members of the PRL family and are the exclusive sources of PL-I, PL-II, PLF, and PLP-E (69,70,72,73,44). PL-I, PL-II, and PLF have been co-localized to trophoblast giant cells (94). The behavior of trophoblast giant cells differs depending upon their intraplacental location and developmental state. Expression patterns involve trophoblast giant cells residing in the choriovitelline placenta and in both the junctional and labyrinth regions of the chorioallantoic placenta. PL-I and PLF first appear immediately postimplantation (73, 75, 95). PL-I terminates at midgestation (11, 73, 75, 95), whereas PLF continues through the second half of gestation at reduced levels of production (16, 70, 75). PL-II expression begins at midgestation and continues until the termination of pregnancy (71-73). Although, PL-I, PLF, and PL-II share cell-specific requirements for expression, they appear to be optimally activated in response to distinct factors in the trophoblast giant cell environment.

Some limited information is available on the regulation of trophoblast giant cell restricted members of the PRL family. *In vivo* manipulations such as hypophysectomy, gonadectomy, and fetectomy influence circulating PL-I and/or PL-II levels (96-103). Removal of the anterior pituitary or ovaries results in an increase in circulating PLs, whereas removal of the fetus decreases serum PL concentrations. Anterior pituitary factors appear to affect PL clearance (104) and ovarian and fetal factors affect the development and organization of the placenta (105). A variety of hormones, growth factors, cytokines, and other activators of signal transduction cascades have been

shown to modulate the production of PL-I, PL-II, and PLF *in vitro* (63, 106). Transcriptional control of PRL family gene expression by trophoblast giant cells has been investigated using transgenic and gene targeted mutant mouse models and

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through the use of a rat trophoblast cell line, Rcho-1, which faithfully mimics many of the features of trophoblast giant cells developing *in situ* (107-110). Investigations have focused on the transcriptional regulation of PL-I, PLF, PL-II, PLP-A, PLP-Cv, and d/tPRP. MAP kinase signaling pathways and transcription factors interacting with AP-1 and GATA elements participate in the control of the PL-I gene (108, 111-113). Consistent with these observations, GATA-2 or GATA-3 null mutant mice exhibit a profound diminution in their expression of PL-I and PLF (112). Putative AP-1 elements and Ets binding sites have also been shown to participate in the regulation of PL-II and d/tPRP gene transcription (93, 114). Additional mapping of DNA regulatory regions controlling trophoblast-specific expression of PLP-A and PLP-Cv genes in Rcho-1 trophoblast cells has been reported (38, 115, 116). Thus far, only 5' flanking DNA associated with the PL-II gene has been reported to effectively target the *in vivo* expression of reporter genes to the placenta (117-119). Significant progress has been made in understanding the control of trophoblast giant cell differentiation and some linkages have been made between pathways controlling trophoblast giant cell differentiation and the expression of members of the PRL gene family (120,121). The basic helix-loop-helix transcription factor, Hand1, plays an essential role in the development of trophoblast giant cells (122). Other proteins with the capacity to modulate Hand1 activities (Id1, Id2, E-factors, Mash2, mSna, and I-mfa) have also been implicated in the control of trophoblast giant cell formation (119, 120,123,124).

### *SPONGIOTROPHOBLAST CELL*

Clearly, the most common expression pattern for members of the PRL family involves dual expression by both spongiotrophoblast and trophoblast giant cells. PLP-B and PLP-F represent exceptions. Within the chorioallantoic placenta PLP-B expression is restricted to spongiotrophoblast cells (66, 72, 125). Similarly in the mouse chorioallantoic placenta PLP-F expression is confined to spongiotrophoblast cells (31, 44). Interestingly, in the rat, PLP-F follows the more typical pattern and is expressed in both spongiotrophoblast and trophoblast giant cell populations (31). Spongiotrophoblast cells first arise at midgestation in association with the formation of the chorioallantoic placenta and at this juncture it appears that their main function is the production of PRL family proteins (89, 126). Hormone/cytokine expression by spongiotrophoblast cells is restricted to the second half of gestation.

Only modest information is available on the regulation of spongiotrophoblast cell production of PRL family members. Retinoic acid attenuates PLP-C and PLP-Iv production by primary cultures of spongiotrophoblast cells (127). There is also some evidence for the separation of DNA regulatory elements controlling spongiotrophoblast versus trophoblast giant cell gene activation within the PLP-Cv promoter (116). The development of spongiotrophoblast cells is dependent upon the helix-loop-helix transcription factor, Mash2 (128), and the epidermal growth factor receptor (129, 130). Consequently, these factors are involved in regulating the expression of PRL family genes, if not directly then indirectly by controlling the formation of the spongiotrophoblast cell.

### *LABYRINTHINE TROPHOBLAST CELL*

The labyrinthine region of the chorioallantoic placenta is chiefly involved in bidirectional nutrient transport between maternal and fetal compartments. However, during the last week of pregnancy, trophoblast cells within the labyrinthine region begin to produce a subset of PRL family members [pL-II, PLF-RP (rat only), PLP-K;

31,49,71]. The cell principally responsible for these activities is situated at the interface with the maternal blood space and appears to be related to the trophoblast giant cell. These labyrinthine trophoblast giant cells are phenotypically distinct from giant cells derived from the Rcho-1 trophoblast cell line which appear to more closely represent junctional zone trophoblast giant cells (31). Labyrinthine trophoblast hormone/cytokine production may facilitate hormone/cytokine entry into the fetal compartment. Similarly, the regulation of labyrinthine PRL family expression is likely directly linked to signals emanating from the fetus.

#### *ENDOVASCULAR TROPHOBLAST CELL*

Trophoblasts throughout the placenta come into direct contact with maternal blood, a property shared with vascular endothelial cells. A subset of rodent trophoblasts, though, form an invasive column that penetrates deep into the deciduum, and surrounds a large maternal blood space to form the "placental vessel" (90). These trophoblasts express several hormones in the PRL family (32), and thus these hormones serve as markers of this invasive cell population. The production of these hormones in the invasive trophoblasts presumably provides an efficient means for their delivery into the maternal circulation, and perhaps also indicates that concentrations of extracellular factors regulating the synthesis of these hormones are high in this region.

#### **TRANSPORT AND TISSUE DISTRIBUTION**

Presently we have a few examples for different modes of hormone delivery. A number of members of the PRL family are present in maternal circulation and a few have been detected in the fetus (106, 132). Cytokine access and availability to target cells can be influenced by posttranslational modifications and interactions with various transport and binding proteins. Glycosylation appears to be the most common posttranslational modification for members of the PRL family. The nature of protein glycosylation appears to be cell type and protein specific (133). Addition of carbohydrate to PL-I by trophoblast giant cells is apparently responsible, at least in part, for its lengthened half-life in circulation relative to the short half-life of the unglycosylated PL-II (4). Specific associations of members of the PRL family with transport or binding proteins have also been observed. Mouse PL-II, in part, circulates bound to  $\alpha_2$ -macroglobulin (134). Transport proteins such as  $\alpha_2$ -macroglobulin can specifically direct ligands to their target cells or effectively protect the maternal environment from the actions of the ligand. In the rat, PLP-A achieves very high concentrations in blood and is known to circulate as a high molecular weight complex bound to another protein(s) (135, 136). The nature of the circulating PLP-A binding protein has not been reported. Some members of the PRL family appear to be restricted in their distribution because of specific interactions with components of the extracellular matrix. D/tPRP binds to heparin containing molecules and is present in the decidual extracellular matrix (137). Such a location is ideal for gaining access to cells that traverse the decidual compartment (trophoblast, immune, endothelial, decidual) and in limiting exposure to extrauterine sites. A few members of the PRL family have been reported to gain access to the fetal circulation, whereas others are selectively excluded from the fetus (132, 138-140). Mechanism(s) responsible for a ligand gaining access to or being excluded from the fetal compartment have not yet been determined but may involve posttranslational modifications or interactions with carrier molecules.

The modes of hormone delivery discussed above are based on only a few members of the PRL family and thus provide us with only a hint of what to expect. We have much to learn about posttranslational processing, tissue distribution, and transport characteristics for the majority of PRL family members.

## FUNCTIONS AND MECHANISMS OF ACTION

Initial investigations into the function of PRL family members were focused on their abilities to mimic the actions of PRL. However, in 1994 it became apparent that members of the PRL family could act on apparently unique cellular targets yielding unique biological responses (141). These observations gave rise to a classification of the PRL family based on function. Members of the PRL family utilizing the PRL receptor signaling pathway were categorized as *classical* and those that did not as *nonclassical*. In the next few paragraphs we outline our current understanding of the function and mechanisms of action for several members of the PRL family.

### *PRL MIMICRY*

Much of PRL family biology grew out of classic observations implicating the rodent uterus and placenta as sources of factors influencing mammary gland development and corpus luteum function. Rodent placental hormones possessing both mammotrophic and luteotrophic actions have been isolated and characterized. PL-I and PL-II stimulate various parameters of mammary epithelial cell growth and differentiation (20, 25, 142, 143) and promote the biosynthesis of progesterone by the corpus luteum (144, 145). PL-I and PL-II are part of a regulatory network that also includes PRL and PL-IV. The expression of these maternal (PRL), trophoblastic (PL-I, PL-IV, and PL-II), and fetal (PRL) hormones are orchestrated in a precise temporal pattern that ensures the presence of activators of the PRL receptor signaling system throughout gestation (7, 83, 84, 146-148). PL-IV is a weak PRL receptor agonist when compared to PRL, PL-I, or PL-II (28, 84). In addition to the mammary gland and corpus luteum, there are several other targets for these ligands in both maternal and fetal compartments. Evidence has arisen for their involvement in the control of maternal behavior (149, 150), the regulation of PRL (151) and insulin secretion (152, 153), and the modulation of immune cells (10, 25, 28, 146, 147). The significance of multiple ligands activating the PRL receptor signaling pathway likely provides for complementarity and presumably some form of specialization (154).

Considerable information has been generated regarding PRL signal transduction (see elsewhere in this volume). However, knowledge about intracellular events subsequent to PRL receptor activation by members of the placental PRL family is minimal. PL-I, PL-II, and PL-IV :- ' (28, 55, 84, 139, 147, 156-159). The only apparent disparity in binding characteristics is the lower affinity of PL-IV for the PRL receptor (28, 84). The reduced biopotency of PL-IV in assays for PRL-like bioactivities (28, 84). It is that PL-I, PL-II, and PL-IV activate signaling pathways downstream of the receptor similar to those activated by PRL. Beyond the abilities of PL-I and PL-IV to Jak2-STAT5 pathway in cellular signaling pathways triggered by PRL, it would appear likely that at least some aspects of PRL signaling are mimicked by PL-II, and PL-IV; however, whether these three placental ligands spectrum of PRL action within their target cells is unknown.

*ANGIOGENESIS*

The ability of hormones in the PRL family to act on endothelial cells and regulate angiogenesis was first shown for a proteolytic fragment of PRL (55). A tantalizing result was obtained for PLF by Nelson Horseman, while testing this hormone for PRL-like bioactivity in the classical pigeon crop sac assay; he found no induction of crop sac growth, but he did see increased vascularization at the site of injection of PLF (141). Subsequent analysis of PLF and PRP indicated that they also have activities on endothelial cells, with PLF stimulating, and PRP inhibiting, endothelial cell migration in cell culture and *in vivo* (141). Since PLF is secreted earlier in gestation than PRP, it seems likely that PLF contributes to the growth of the placental vasculature and PRP later limits that growth (161). Consistent with these roles, the mid-gestation placenta secretes net angiogenic activity, most of which is removed with an antibody against PLF, whereas the later gestation placenta secretes anti-angiogenic activity, most of which is blocked by an antiserum against PRP (141). Furthermore, disruption of the gene encoding the GATA-2 transcription factor, which is essential for PLF expression *in vivo*, results in a significant decrease in mid-gestation placental secretion of angiogenic activity and in a decrease in neovascularization at the implantation site (112).

PLF is able to bind to endothelial cells at the implantation site and in the fetus, as well as to some non-endothelial cell types in the fetus, including developing vertebrae (140, 141, 162). Lee and Nathans (21) had reported that this hormone interacts with the insulin-like growth factor II/mannose 6-phosphate receptor (IGF-II/M6PR), and that this interaction depends on a mannose 6-phosphate modification to PLF's N-linked carbohydrate. In contrast, Nilsen-Hamilton and colleagues (163), found that PLF (which they refer to as mitogen regulated protein) binds a distinct receptor in the uterus that does not involve mannose 6-phosphate. To determine which, if either, of these receptors is relevant for PLF binding to endothelial cells, the ability of mannose 6-phosphate to block hormone binding and action was examined; this phosphorylated sugar was found to compete for PLF binding sites on endothelial cells, to inhibit PLF-induced endothelial cell migration in culture, and to prevent PLF-stimulated neovascularization *in vivo* in a corneal implant assay (162). Thus, the IGF-II/M6PR appears to be a relevant component of the binding and signaling complex in PLF-regulated angiogenesis.

Despite the ability to bind IGF-II, a potent activator of cell proliferation, the IGF-II/M6PR seems to be incapable of transmitting signals that lead to DNA synthesis and cell cycle progression; this growth factor function of IGF-II is instead mediated by binding of the factor to the IGF-I receptor tyrosine kinase (164). The effects of PLF on endothelial cells, though, implicate a distinct signaling pathway that leads to cytoskeletal reorganization and cell migration. The IGF-II/M6PR may itself signal in this pathway, or upon PLF binding may interact with a signaling receptor protein as part of a larger complex. An event that occurs downstream of PLF binding to the IGF-II/M6PR receptor includes a weak activation of MAP Kinase through a pertussis toxin-sensitive step, suggesting that PLF signaling is mediated by an inhibitory G protein that communicates with the MAP Kinase pathway; activation of this pathway is required for PLF-induced endothelial cell migration (165). ~

The discovery that PLF is both angiogenic and expressed in a growth-regulated manner in mouse fibroblast cell lines (12, 166) suggests that reexpression of this placental angiogenic hormone might also occur under certain conditions outside of the pla-

centa. Fibroblasts are intimately involved in wound healing, a process that includes the growth of blood vessels, but no PLF gene reactivation was detected in fibroblasts at wound sites (J. Groskopf and D. Linzer, unpublished results). Another possibility is that PLF expression may recur in tumor cells as they progress from small, non-angiogenic masses to aggressive and metastatic cancers; some mouse tumor models do show PLF expression (167, 168), but it is not yet known what contribution PLF makes to the growth of these or other tumors.

In contrast to the presumed stimulatory effect of PLF on tumor growth by increasing tumor vascularization, the anti-angiogenic hormone PRP would be predicted to restrict tumor growth by reducing tumor vascularization. Indeed, tumor cells engineered to secrete PRP are significantly reduced in their growth *in vivo*, but are unaffected in their growth in culture where a blood supply is irrelevant (N. Bengtson and D. Linzer, manuscript submitted). The identification of PRP in rodents other than the mouse, including the hamster (169) and rat (31,32), suggest that the effects of PRP may be conserved. The mechanism by which PRP blocks angiogenesis is not known; neither the receptor nor any downstream signaling components have yet been implicated.

### *HEMA TOPOIESIS*

As members of the PRL family, these placental hormones are also in the cytokine superfamily, and as such may be expected to act in hematopoiesis. In mammalian reproduction, maternal blood volume increases (170), and as a result proliferation and differentiation in the hematopoietic system must increase to maintain the concentrations of the many types of blood cells. Differentiation of one of these cell types, megakaryocytes (MK cells), involves numerous rounds of DNA synthesis without cell division until the cell fragments into blood platelets, which in turn mediate wound and vascular repair. Platelet levels increase in pregnant rodents (171), which suggests that increased levels of cytokines that act on MK cells are present.

One cytokine that targets MK cells during pregnancy is PLP-E (172). PLP-E binds to a specific receptor on MK cells and stimulates primary MK cell differentiation, both in terms of increased cell size and increased DNA content. In combination with interleukin 3 (IL-3), a cytokine that stimulates the proliferation of MK cell precursors (colony forming unit-megakaryocyte, or CFU-MK), PLP-E enhances colony formation in semi-solid culture, suggesting that PLP-E acts to stimulate CFU-MK proliferation, to improve CFU-MK survival, or to promote the commitment of multi potential precursors to the MK lineage. PLP-E's effects are similar to those of the IL-6 family of cytokines, although addition of IL-3, PLP-E, and IL-6 together has an even greater effect on CFU-MK colony formation than IL-3 with either of the other factors alone. Another similarity is that both PLP-E and IL-6 activate a signal transduction pathway that requires the signaling co-receptor gp130.

The action of PLP-E is not restricted to MK cells, as this hormone is able to stimulate the formation of granulocyte/macrophage cell colonies (CFU-GM) in primary bone marrow cell cultures (173). The ability of PLP-E to act on both CFU-MK and CFU-GM suggests that this hormone may target a common, myeloid precursor cell. The actions of PLP-E may overlap with PLP-F, which is expressed later in gestation. PLP-F and PLP-E appear to compete for the same receptor on MK cells (J. Lin, H. Lum, and D. Linzer, unpublished results), but it is not yet known if PLP-E and PLP-F function as agonists or antagonists.

### IMMUNE CELL MODULATION

PRL is a known modulator of immune cell function (see elsewhere in this volume). In this section we present evidence for the interaction of other PRL family members with components of the immune system during pregnancy.

Hemochorial placentation, as occurs in both primates and rodents, results in the establishment of a close connection between maternal and fetal tissues (90). The close connection facilitates the exchange of nutrients and wastes at the expense of an increased risk of attack by the maternal immune system. This is of special relevance in that the uterine stroma is an abundant reservoir of immune cells (174). Trophoblast and decidual cells and their secretory products likely provide the signaling system that coordinates activities within the uterine immunologic milieu. The nature of the controls on the maternal immune system must be transitory and local (intrauterine); otherwise they may compromise the mother's ability to respond to infections. Through the use of an alkaline phosphatase (AP)-ligand tagging strategy, eosinophils and natural killer (NK) cells have been identified as targets for two members of the PRL family, d/tPRP (175) and PLP-A (176), respectively.

### D/TPRP-EOSINOPHIL INTERACTIONS

D/tPRP was originally isolated during a search for a decidual luteotropin (41). Although, d/tPRP exhibits significant structural similarities to PRL, it does not interact with PRL receptors and thus its putative luteotropic actions are suspect (137, 175). D/tPRP is predominantly expressed by decidual cells during the first half of pregnancy and subsequently by spongiotrophoblast and trophoblast giant cells of the chorionic-lamellar placenta during the second half of pregnancy (34, 41, 42, 67, 85). Once secreted from its cellular sources d/tPRP readily associates with heparin containing molecules and accumulates in the extracellular matrix (137, 175). Thus, d/tPRP is in an excellent position to interact with various populations of cells traversing the uteroplacental compartment, including immune cells. Additionally, these heparin-binding attributes also restrict d/tPRP's actions to the uterine compartment. In a xenotransplantation model, CHO cells expressing d/tPRP more readily form tumors in athymic mice than do control CHO cells (137). The *in vivo* growth differences in the two CHO cell populations cannot be accounted for by *in vitro* growth rates and are independent of effects of d/tPRP on the host vasculature. These observations suggested that d/tPRP participates in the regulation of host immune cell responses. More recent insights regarding d/tPRP have come from the use of an AP-d/tPRP fusion protein. Using this approach, d/tPRP has been shown to interact with eosinophils (175).

Eosinophils contribute actively to inflammatory responses through their secretion of a variety of chemical mediators (177). These cells are actively recruited to sites of allergic responses (178), tumors (179), and parasite infestation (180). Eosinophil dynamics are precisely controlled within the uterus by the hormonal milieu, and exhibit dramatic fluctuations during the reproductive cycle and pregnancy (181-183). For example, estrogen stimulates the infiltration of eosinophils into the uterus, whereas uterine exposure to progesterone during pregnancy results in eosinophil exit and death (184, 185). Since d/tPRP is a product of the progesterone-dependent decidua and it specifically interacts with eosinophils, this cytokine is a candidate mediator of the anti-inflammatory actions of progesterone during gestation. The nature and mechanism of d/tPRP effects on eosinophil function have not yet been described.

## Chapter 8

### *PLP-A AND NK CELLS*

PLP-A was one of three cDNAs, structurally related to PRL, originally identified during a search for the PL-II cDNA (33). Expression of PLP-A protein and mRNA are restricted to spongiotrophoblast cells and trophoblast giant cells of the junctional zone of the rat and mouse chorioallantoic placenta (34, 35, 71, 72): Although, PLP-A has significant structural homologies to PRL it is not capable of binding to PRL receptors or activating the PRL receptor signaling pathway (136).

The generation of AP-PLP-A fusion proteins led to the identification of specific PLP-A binding to NK cells within the mesometrial compartment of the uterus from pregnant rats and mice (176). These observations were supported by the co-distribution of PLP-A targets with cells expressing the rat NK cell surface marker, gp42, the absence of PLP-A binding in conceptuses from NK cell deficient tge26 mice, and the specific interaction of PLP-A with rat NK cells. Furthermore PLP-A effectively suppresses rat NK cell cytolytic activities. Uterine NK cells have been proposed to participate in immunological adjustments to pregnancy and in the establishment of the chorioallantoic placenta (174, 186). During pregnancy, NK cells undergo considerable morphological and functional changes creating, in effect, an NK cell with a unique phenotype. Midgestation uterine NK cells are distinct in their bio-effector secretory profile (cytokines and nitric oxide) and their relative absence of cytolytic activities (174, 186).

PLP-A represents an intriguing paracrine signal participating in a new paradigm of embryonic-maternal communication between trophoblast cells and uterine NK cells. Suppression of NK cell killing activity facilitates survival of genetically disparate extraembryonic and embryonic tissues. NK cells also release nitric oxide which directly influences uterine vasculature (187, 188), facilitating the delivery of nutrients to the developing placenta, while their elaboration of cytokines (189) potentially promotes the growth and maturation of the chorioallantoic placenta. Whether PLP-A influences these latter aspects of uterine NK cell function remain to be determined. Effects of PLP-A on extrauterine NK cells are likely obviated by the association of PLP-A with serum binding proteins (135, 136). NK cell surface targets and intracellular signaling pathways activated by PLP-A have not been reported.

### *SPECULATIONS ABOUT OTHER PRL FAMILY MEMBERS*

We have only scratched the surface in understanding the biology of the PRL family. Currently we have an appreciation of the actions or cellular targets for ten of the approximately two dozen currently identified members (Table 8-4). Although the present state of our knowledge is fragmentary, it is sufficient to recognize that we have a very compelling biological story. Members of the PRL family are being used to enlist maternal tissue responses that directly benefit prenatal and postnatal development.

Over the next few years, we will gain new insights into the biology of each member of the PRL family. As our analyses expand, we are likely to discover additional maternal targets and extraembryonic and/or fetal tissue responses dependent on members of the PRL family. The AP-tagging strategy will provide an objective means of identifying cellular targets for orphan members of the PRL family. Gene targeting represents a complementary approach to directly evaluate the significance of each family member in the physiology of pregnancy. The extensive specialization among PRL family members regarding structure, expression patterns, and/or cellular targets portends well for the identification of phenotypes in null mutant mice. Receptor signaling pathways respon-

**Table 8-4. TARGET TISSUES FOR MEMBERS OF THE PRL FAMILY**

<b>Hormone</b>	<b>Cellular targets</b>	<b>Refs</b>
PRL, PL-I, PL-II	Mammary gland, ovary, liver, pancreas, brain, T-cells	25, 139, 144, 149-153, 190, 191
PL-IV	Ovary, liver, T-cells	28, 84
PLF	Endothelial cells, uterine cells, muscle cells	141, 163, 192, 193
PRP	Endothelial cells	141
PLP-A	Natural killer cells	176
PLP-E, PLP-F	Hematopoietic precursors, megakaryocytes	172, 194
D/tPRP	Eosinophils, trophoblast	175

sible for transducing each ligand's action will also be resolved. At present, it is not clear whether specific receptor signaling systems have co-evolved with each of the PRL family members~ 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 the future, we may observe examples of each of these possibilities.

## EVOLUTIONARY CONSIDERATIONS

Even a cursory analysis of the PRL family opens interesting evolutionary issues. Members of the PRL family have been identified in several species (195) and significant molecular information exists in a few rodent species (rat, mouse, hamster; 154), a few ruminants (cow, sheep, goat; 196, 197), and a couple of primate species (human, rhesus monkey; 198). Unlike PRL, whose structure, expression patterns, and functions have been relatively well conserved, it has been difficult to discern species conservation for other members of the PRL family. Orthologous relationships are apparent within closely related species (e.g. mouse versus rat; cow versus sheep) but are not evident when examining more distantly related species (e.g. rodent versus ruminant versus primate). Some species are notable for their use of different ancestral templates, while others are notable for their apparent lack of more than a single member of the PRL family. Key among the evolutionary issues related to the PRL family is the apparent species diversity.

### *SPECIES DIFFERENCES ARE APPARENT BUT ARE THEY REAL?*

#### **Ancestral gene templates**

The PRL family arose by duplication from ancestral genes (199). Two different templates have been used for the generation of PRL related pregnancy-specific regulators. Primates evolved a family of proteins expressed in the placenta more closely related to GH, while rodents and ruminants used an ancestral PRL related gene as an evolutionary prototype. The utilization of GH as a template in primates has a functional corollary. Primate GHs are unique among GHs in their ability to activate the PRL receptor signaling pathway (200). Whether primates also possess a family of proteins more closely related to PRL is not known. The evolution of protein families from PRL ancestral prototypes for the purpose of regulating pregnancy must be related to the well-recognized functional versatility of PRL (201). Over one hundred different biological actions have been

attributed to PRL. The structural motifs within the PRL backbone responsible for this versatility are not well understood.

Absence of placental lactogenic activities in some species

Some species such as the pig, horse, rabbit, cat, and dog do not appear to possess a classic PL (195). This may represent divergent evolution or alternatively it may relate to limited experimentation. Some of the apparent non-expressing species may actually possess a classic PL with a restricted expression pattern that has not been adequately tested. Alternatively, nonclassical members of the PRL family may be expressed by uteroplacental tissues in these species. In fact, the driving force(s) for derivation of the PRL family may relate to more elemental aspects of viviparity, such as vascular connectivity and maternal-fetal tolerance rather than the regulation of ovarian or mammary gland function.

**Deficits in our research** Our present information about the PRL family is biased by the small group of species studied and the obstacles associated with studying pregnancy-related events in some species. The mouse and the rat are particularly tractable experimental models, especially in relation to the duration of their gestation. Research in the mouse and rat has further benefited from the mouse and rat genome projects and the availability of ESTs from an assortment of uterine and extraembryonic cDNA libraries representing a variety of different phases of pregnancy. Thus the absence of establishing true cross-species homologies may be real or the result of our limited current state of knowledge. Over the past few years, we have accumulated many new tools for evaluating PRL families. Consequently, there is considerable merit in revisiting a comparative approach. Completion of existing genome projects and expansion of genome characterization to other species will also help resolve these uncertainties.

What are the implications of the apparent species diversity?

It is essential that we place the PRL family in proper context. As is evident from the preceding discussion, the PRL family is a collection of hormones/cytokines that regulate pregnancy. Problems associated with young developing within the female reproductive tract are similar for all species. Adequate supplies of nutrients must be delivered to the embryo/fetus without compromising the mother. However, the solutions utilized by individual species vary widely. Among viviparous species there are striking differences in the organization of the maternal-fetal interface, the length of gestation, and the progression of embryonic/fetal development. Hence, it is not surprising that factors controlling the gestational state differ in a species-dependent manner. The PRL family represents a newly evolved collection of regulators directly linked to viviparity. Functional homologies among species will exist and may include the ligands, their cellular targets, and/or components of their signaling pathways.

## **DEVELOPMENT OF NEW THERAPEUTIC STRATEGIES**

An exciting possibility is that rodent PRL-related hormones will reveal new mechanisms in the regulation of physiology, an expectation suggested by the unusual physiological changes that occur during pregnancy and the importance of the uterus and placenta in the endocrine control of pregnancy. If new molecular targets in signal transduction or new ways to combine known signaling components are revealed by analyzing the mechanisms of action of the uteroplacental hormones, then presumably

these hormones may become valuable tools in the design of new clinical therapies. For *ex vivo* therapies, for example in the expansion of human bone marrow in cell culture for autologous transplantation, novel hematopoietic effects of these mouse hormones might be exploited without concern for the delivery or immunological problems that would occur if these proteins were to be administered *in vivo*. For clinical interventions requiring *in vivo* treatment, for example in controlling autoimmune effects caused by NK cells or in restricting tumor growth by preventing tumors from attaining a blood supply, hormones such as PLP-A and PRP may help to identify receptors, and these receptors could then be used to screen small molecule libraries for ligands which would be more compatible with use in humans. 0

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