

# Prolactin family miniarray: a tool for evaluating uteroplacental–trophoblast endocrine cell phenotypes

G. Dai\*, L. Lu, S. Tang, M. J. Peal and M. J. Soares†

*Institute of Maternal–Fetal Biology, Departments of Molecular and Integrative Physiology and Obstetrics and Gynecology, The University of Kansas Medical Center, Kansas City, KS 66160, USA*

The prolactin family represents a group of hormones and cytokines that participate in the control of maternal and fetal adaptations to pregnancy. The aim of this study was to develop a simple assay for monitoring patterns of prolactin family gene expression in rats and mice. Prolactin family cDNAs were spotted on to nylon membranes. Total RNA was extracted from tissues or cells. cDNAs were generated, radiolabelled using reverse transcriptase, and used as probes to hybridize with the prolactin family miniarrays. Pituitary, uterine decidual tissue and placenta each expressed a unique profile of prolactin family members.

Placental tissues exhibited regional- and temporal-specific patterns of expression. Prolactin family gene expression differed markedly in mid-pregnant versus late gestation placental tissues and between the junctional and labyrinthine zones of the chorioallantoic placenta. Marked changes in prolactin family gene expression were also observed in cultured trophoblast cells undergoing differentiation. In conclusion, the prolactin family miniarray assay is an effective method for evaluating the endocrine phenotype of the uterus, placenta and trophoblast cells.

## Introduction

The uterus and the placenta influence the maternal–fetal interface by their production of hormones and cytokines. Among the various regulatory signals secreted by the uterus and placenta is a prominent family of proteins related to pituitary prolactin and known as the uteroplacental prolactin family (Soares and Linzer, 2001). At least 19 different ligands bearing some similarity to prolactin have been identified in mice and rats, including placental lactogens (PLs), prolactin-like proteins (PLPs), prolactin-related protein (PRP), proliferin and proliferin-related protein (PLF-RP). This gene family encodes proteins with a diverse array of biological actions implicated in the establishment and maintenance of pregnancy (Soares and Linzer, 2001). These actions include the regulation of ovarian and mammary gland function and inflammatory, immune, endothelial and hematopoietic cell development and function. There are three major types of cell involved in the biosynthesis of members of the uteroplacental prolactin family in rats and mice: (i) lactotrophs of the anterior pituitary; (ii) decidual cells of the uterus; and (iii) trophoblast cells of the placenta (Soares *et al.*, 1991, 1996, 1998). Members of the prolactin family are expressed in cell- and temporal-specific patterns in the uteroplacental compartment and anterior pituitary (Soares and Linzer,

2001). In this report, a simple array-based hybridization assay for monitoring gene expression of the mouse and rat prolactin gene family is described. The assay permits the phenotyping of uteroplacental tissues and trophoblast cells.

## Materials and Methods

### *Animals and tissue dissections*

Holtzman rats were obtained from Harlan Sprague–Dawley Inc. (Indianapolis, IN). CD-1 mice were purchased from Charles River Laboratories Inc. (Wilmington, MA). Animals were housed in an environmentally controlled facility, with lights on from 06:00 to 20:00 h and were allowed free access to food and water. Timed pregnancies were generated by cohabitation of female and male animals. The presence of a copulatory plug or spermatozoa in vaginal smears was designated day 0 of pregnancy in rats. Rat uteroplacental tissues were dissected as described by Faria *et al.* (1990). The presence of a copulatory plug was designated day 1 of pregnancy in mice. Mouse uteroplacental tissues were dissected as described by Orwig *et al.* (1997) and Dai *et al.* (1998). The University of Kansas Animal Care and Use Committee approved protocols for the care and use of animals.

### *Rcho-1 trophoblast cell line*

Elucidation of regulatory networks controlling trophoblast cell differentiation has been facilitated by the availability of the rat Rcho-1 trophoblast cell line (Faria and Soares, 1991; Peters *et al.*, 2000). Rcho-1 trophoblast cells

\*Present address: Department of Internal Medicine, The University of Texas Medical Branch at Galveston, 301 University Boulevard, TX 77555–1064, USA

†Correspondence

Email: msoares@kumc.edu

can be manipulated to proliferate or to differentiate along the trophoblast giant cell lineage (Faria and Soares, 1991; Hamlin *et al.*, 1994; Peters *et al.*, 2000). The Rcho-1 trophoblast cell line was routinely maintained in subconfluent conditions with RPMI-1640 culture medium supplemented with 20% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) as reported by Faria and Soares (1991), Hamlin *et al.* (1994) and Peters *et al.* (2000). Differentiation was induced by growing cells to near confluence in FBS-supplemented culture medium and then replacing the medium with NCTC-135 culture medium supplemented with 10% horse serum (HS; JRH Biosciences). High cell density and the absence of sufficient growth stimulatory factors (removal of FBS) facilitate trophoblast giant cell formation (Hamlin *et al.*, 1994; Hamlin and Soares, 1995; Peters *et al.*, 2000).

### *Trophoblast stem cells*

Trophoblast stem (TS) cells represent a population of cells derived from the mouse blastocyst with the capability to differentiate into all trophoblast cell lineages (Tanaka *et al.*, 1998). TS cells were obtained from J. Rossant, Mount Sinai Hospital (Toronto, Canada). Rossant and co-workers have previously demonstrated that heparin and fibroblast growth factor 4 (FGF-4) stimulate proliferation of TS cells and inhibit differentiation (Tanaka *et al.*, 1998). TS cells were maintained in heparin-FGF-4 supplemented culture medium comprising 30% TS medium (RPMI-1640 supplemented with 20% FBS, 1 mmol sodium pyruvate  $\text{l}^{-1}$ , 50  $\mu\text{mol}$  2-mercaptoethanol  $\text{l}^{-1}$ ) and 70% mouse embryonic fibroblast conditioned medium as described by Tanaka *et al.* (1998). Heparin and FGF-4 were obtained from Sigma Chemical Co. (St Louis, MO) and added to final concentrations of 1  $\mu\text{g ml}^{-1}$  and 25 ng  $\text{ml}^{-1}$ , respectively. These culture conditions promoted the optimal proliferation of TS cells (Tanaka *et al.*, 1998). Culturing the cells in culture medium devoid of FGF-4, heparin and embryonic fibroblast conditioned culture medium induces trophoblast differentiation (Tanaka *et al.*, 1998).

### *Preparation of prolactin family DNA miniarrays*

Miniarray assays for the mouse and rat prolactin families have been established. Each of the cDNAs corresponding to the mouse and rat prolactin gene families were acquired from Research Genetics (Huntsville, AL); American Type Culture Collection (Rockville, MD); D. I. H. Linzer (Northwestern University, Evanston, IL) or K. Shiota (University of Tokyo), or were cloned by our laboratory (Tables 1 and 2). Oligonucleotide primers were designed that specifically amplify cDNAs corresponding to the nucleotide sequences encoding the mature proteins. Nucleotide regions corresponding to 5' and 3' untranslated regions and signal peptides were excluded. Additional DNAs, including salmon sperm DNA and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as negative and positive controls, respectively. cDNAs were amplified using the polymerase chain reaction (PCR) with corresponding oligonucleotide primers

(GenoMechanix, Alachua, FL; Tables 1 and 2) and prolactin family cDNA templates. Plasmid DNA (5 ng) was used in 100  $\mu\text{l}$  PCR reaction containing 1  $\times$  PCR buffer (Life Technologies, Rockville, MD), 1.5 mmol  $\text{MgCl}_2 \text{l}^{-1}$ , 1 mmol dNTPs  $\text{l}^{-1}$ , 0.2  $\mu\text{mol l}^{-1}$  each primer and 5 units of Taq DNA polymerase (Life Technologies). Initial denaturation was at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50 to 65°C for 1 min and extension at 72°C for 1 min, followed by the final extension at 72°C for 3 min. Aliquots of these PCR products were separated by electrophoresis on 1.5% agarose gels to verify their size and purity. PCR products were purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA), and then quantified with a spectrophotometer at an absorbance of 260 nm. PCR products were denatured by boiling for 5 min and immediately chilling on ice. Purified PCR products (20 ng per well) were spotted on nylon filters (Schleicher & Schuell, Keene, NH) with a dot blot apparatus (Bio-Rad, Hercules, CA).

### *RNA isolation and labelling*

Total RNA was extracted from tissues or cultured cells using the Trizol reagent (Life Technologies; Chomczynski and Sacchi, 1987). RNA samples were quantified by spectrophotometry and then separated by electrophoresis in 1% agarose to determine their integrity. Total RNA was labelled according to Mammalian GeneFilters Microarrays Protocol (Research Genetics). Total RNA (10  $\mu\text{g}$  per sample) was primed with 2  $\mu\text{g}$  oligo dT (Research Genetics) in 10  $\mu\text{l}$  total volume by heating at 70°C for 10 min and then chilling briefly on ice. Elongation reactions were performed in 1  $\times$  First Strand Buffer (Life Technologies), 3.3 mmol DTT  $\text{l}^{-1}$  (Life Technologies), 1 mmol  $\text{l}^{-1}$  each of dATP, dGTP, dTTP (Amersham Pharmacia Biotech, Piscataway, NJ), 300 units of Superscript II Reverse Transcriptase (Life Technologies) and 3.3 mCi [ $\alpha$ - $^{32}\text{P}$ ] per mmol dCTP per litre (PerkinElmer Life Science, Boston, MA) in a total volume of 30  $\mu\text{l}$  at 37°C for 90 min. Probes were purified with micro Bio-Spin 30 chromatography columns (Bio-Rad) and then boiled for 5 min before use.

### *Hybridization and analysis*

Prehybridization was performed at 42°C for 4 h in a prehybridization solution containing 50% formamide, 6  $\times$  sodium chloride sodium phosphate EDTA buffer (SSPE), 5  $\times$  Denhardt's reagent, 0.5% SDS and 100  $\mu\text{g}$  salmon sperm DNA  $\text{ml}^{-1}$ . Hybridizations were carried out at 42°C overnight by adding probe to the prehybridization solution. Membranes were washed twice for 15 min each in 2  $\times$  SSPE and 0.5% SDS at 42°C followed by stringent washing in prewarmed 0.1  $\times$  SSPE and 0.1% SDS at 68°C for 30 min. Membranes were dried and exposed to X-ray film (Eastman Kodak Co., Rochester, NY) at -80°C or placed in PhosphorImager cassettes (Amersham Pharmacia Biotech) and quantified using the ImageQuant software (Version 4.2A, Molecular Dynamics, Sunnyvale, CA).

**Table 1.** Primer sets used for members of the mouse prolactin gene family

Gene	GenBank accession number	Forward primer	Reverse primer	Product size (bp)
Prolactin	X02892	5' CCTCTGCCAATCTGTCCGCT 3'	5' TTAGCAGTTGTTTTGATGGGC 3'	596
PL-I	M35662	5' GCCCACTGAAGACCTGTATAC 3'	5' TGAAGACAACCTCGGCACCTC 3'	554
PL-II	M14647	5' TATCGATTACCCACTGAAAAGC 3'	5' TTATGAACATCACGACACTTC 3'	553
Proliferin	K02245	5' ATGATTTTGGATGCCTGGGAA 3'	5' AACATGTAACACTTCAGGACG 3'	313
Proliferin-RP	X02594	5' GTGCCTCTGCACCCATAAT 3'	5' AAATTCAGAGTAGCAGACATC 3'	650
PLP-A	AF015562	5' GCTAAAAGGTTGAATGTCCAC 3'	5' GCAGTTGCTTTGGATCAGTCG 3'	581
PLP-B	AF015563	5' GTGCCCATGTATGCCAGTTTG 3'	5' GCACTTCAAAGCCTGATACT 3'	578
PLP-C $\alpha$	AF090140	5' AATCTTCCATGTGTTGCAGAA 3'	5' GCAATCTTTCCAGTTATGAG 3'	629
PLP-C $\beta$	AF158744	5' CATGCAGACAGCTGGAACCTC 3'	5' ACATTTCCAAAACAGTCAGGG 3'	604
PLP-C $\gamma$	AF466150	5' GCATGTATGGCAGAAAAGAGC 3'	5' ACAGCTCTGCAATGAACCTTT 3'	687
d/tPRP	AF015729	5' TGAATGTCAAACAGGAGAGAA 3'	5' CAATCTTGCCCAGTTATGCGG 3'	626
PLP-E	AF020525	5' CTACCTTTAAGCAGCAATGAT 3'	5' AGTTTTGGAAGATAAGCAGCT 3'	695
PLP-F	AF020524	5' AGCAATGAGACTGATGGTTAT 3'	5' TGAAGCATCTCTAATATTTTTGGA 3'	656
PLP-J	AB019118	5' TATGACCGGAAATCCAATGAA 3'	5' GGTTTTGATTTTGCCATGCTT 3'	536
PLP-K	AY077703	5' TGGTCTGGACTGGCATCTTTG 3'	5' AGTACTTCCAAGCAATTTTTATTT 3'	259
PLP-L	AF226611	5' ATCATGGAAGATGGAGTTGC 3'	5' GCACAATTTGGTCTCTCAGGA 3'	579
PLP-M	AF234636	5' CCCATATGCTCAGTAAGGAAT 3'	5' TTTGCATTTACCATCTTGAG 3'	572

PL, placental lactogen; proliferin-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual-trophoblast prolactin-related protein.

**Table 2.** Primer sets used for members of the rat prolactin gene family

Gene	GenBank accession number	Forward primer	Reverse primer	Product size (bp)
Prolactin	V01249	5' CTGTTCTGGTGGCGACTGC 3'	5' GTTTTTATGGACAATTTGGCACCTC 3'	576
PL-I	D21103	5' TCCAAACCAACTGCCATTGTGTCC 3'	5' GACTCTCCACTCATTCTTAAAGAC 3'	595
PL-Iv	U32679	5' TCCAAACCAACTGTGCTTGTGTCC 3'	5' AACTCATTCTTAAAGACAACCTCGGCA 3'	584
PL-II	M13749	5' GCACCAAATTACCGAATGTCCACT 3'	5' TCAGCAGTTGTTGTTATAAATATCGCG 3'	575
Proliferin-RP	AF139809	5' ATAAATGCCAGCGAGACTACCCTC 3'	5' TCAAAATCTGAGTAGCAGACATTATC 3'	641
PLP-A	M13750	5' ATGAGAGCTAAACTGTTGAATGTC 3'	5' GCACTTGCTTCGGATCAGTC 3'	587
PLP-B	M31155	5' AGTGGGGCTGGGCGTGGTGA 3'	5' TCTATTATAGATAAGTCTACACTTCAGTAG 3'	581
PLP-C	M76537	5' GCATGTATGGTGAAGACGGT 3'	5' GCAATCTTTCCAGTTATGAGACA 3'	617
PLP-Cv	U93351	5' GCATGTATGGTGAAGACGGT 3'	5' GCAATCTTTCCAGTTATGAGACA 3'	617
PLP-D	AB000107	5' CTTGCGGAAGAGGGCGGCT 3'	5' GCAATCTTTCCAGTTGTGAGACA 3'	620
PLP-H	AB009889	5' GCATGTATGGTGAAGAGGGT 3'	5' GCAATCTTTCCAGTTATGAGACA 3'	617
d/tPRP	L06441	5' CTGCTTGTACCTGGAAGAA 3'	5' TTCCTTCCCTGTTATGCGACACAT 3'	626
PLP-F	AF139808	5' AATAGCAATGAGACTGATGGCGAT 3'	5' AGAATCTTCACTATTGATGGATAAGCA 3'	650
PLP-I	AB019791	5' TGTTCAATGAAGATCTCTTGACC 3'	5' ATTATGAATGATACGGCACTTTAGG 3'	548
PLP-J	AB019945	5' ACACCATATGACCAGATGTCCAAT 3'	5' CCACTTGTTTTAATAATGTTAGGCC 3'	545
PLP-K	AF234635	5' ATATCCACCTGTGCAGAGAG 3'	5' GCAGTTGTTTTGATTGAATATTTGGTA 3'	590
PLP-L	AB022883	5' TGCCAGAAGTCTCAACTAC 3'	5' TTCATTAGGCACAATTTGTTTCTCAG 3'	572
PLP-M	AF226608	5' TGCTTAGTAAGGAATGGGCGCTGC 3'	5' GCACTTACCATCTTGAGAGCAGTTTC 3'	578
Growth hormone	V01237	5' TCCAGTCTGTTTGCCAATGCTGTG 3'	5' AAAGCGGCGACACTTCTAGACC 3'	530

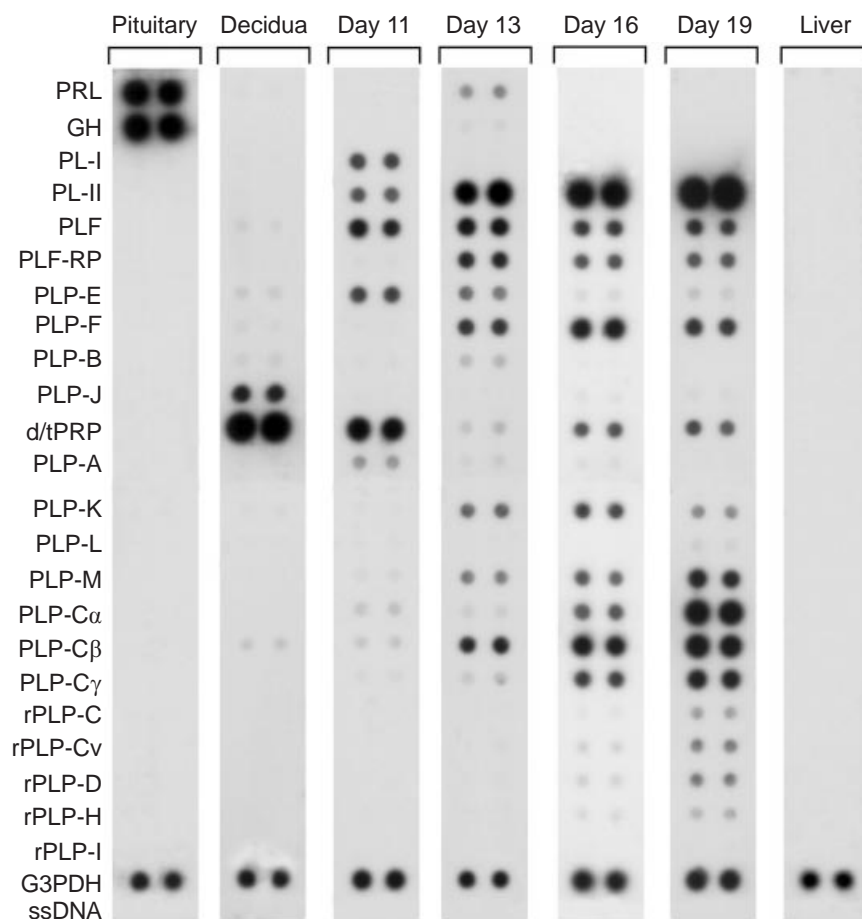
PL, placental lactogen; proliferin-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual-trophoblast prolactin-related protein.

### Statistical analysis

The data were analysed by analysis of variance. The source of variation from significant *F* ratios was determined with the Newman–Keuls multiple comparison test (Keppel, 1973).

### Results

The prolactin gene families of the mouse and rat consist of numerous members expressed in key tissues associated with pregnancy. These features were used to develop a hybridization-based assay for monitoring uteroplacental



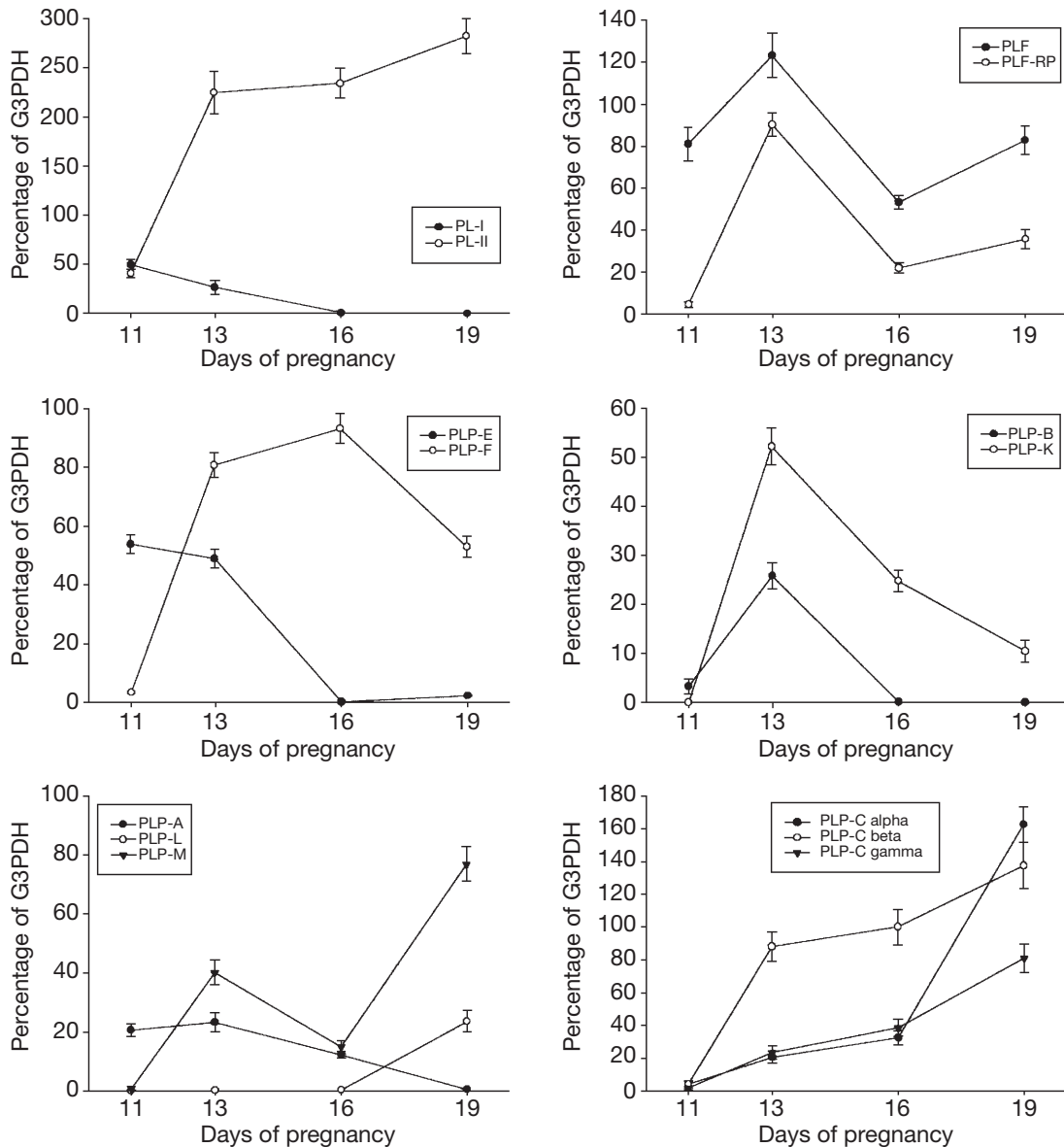
**Fig. 1.** Evaluation of mouse anterior pituitary, decidual and placental phenotypes with the prolactin (PRL) family miniarray assay. cDNAs for all members of the mouse PRL gene family and five unique members of the rat PRL gene family (rPLP-C, rPLP-Cv, rPLP-D, rPLP-H, rPLP-I) were isolated, characterized and spotted on to nylon membranes. Total RNA was prepared from mouse anterior pituitary, decidua from day 8 of gestation and placental tissues from day 11, day 13, day 16 and day 19 of gestation, and prolactin family expression assessed with the PRL family miniarray assay. Liver served as a control tissue for the procedure. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as an internal positive control. Salmon sperm DNA (ssDNA) was a negative background control for hybridization. Data presented in this figure are representative of at least three different experiments. GH, growth hormone; PL, placental lactogen; PLF, proliferin; PLF-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual-trophoblast prolactin-related protein.

tissue and trophoblast cell prolactin family endocrine phenotypes.

#### *Analysis of uteroplacental tissue phenotypes with the prolactin family miniarray assay*

Prolactin family miniarrays were used to examine gene expression patterns in the anterior pituitary, uterine decidua and placental tissues of mice (Figs 1 and 2). Prolactin and growth hormone mRNAs were abundantly expressed in the anterior pituitary. Uterine decidual tissue expressed decidual-trophoblast PRP (d/tPRP) and PLP-J, whereas placental tissues showed a dynamic pattern of prolactin family gene activation (Figs 1 and 2). Placental expression patterns are

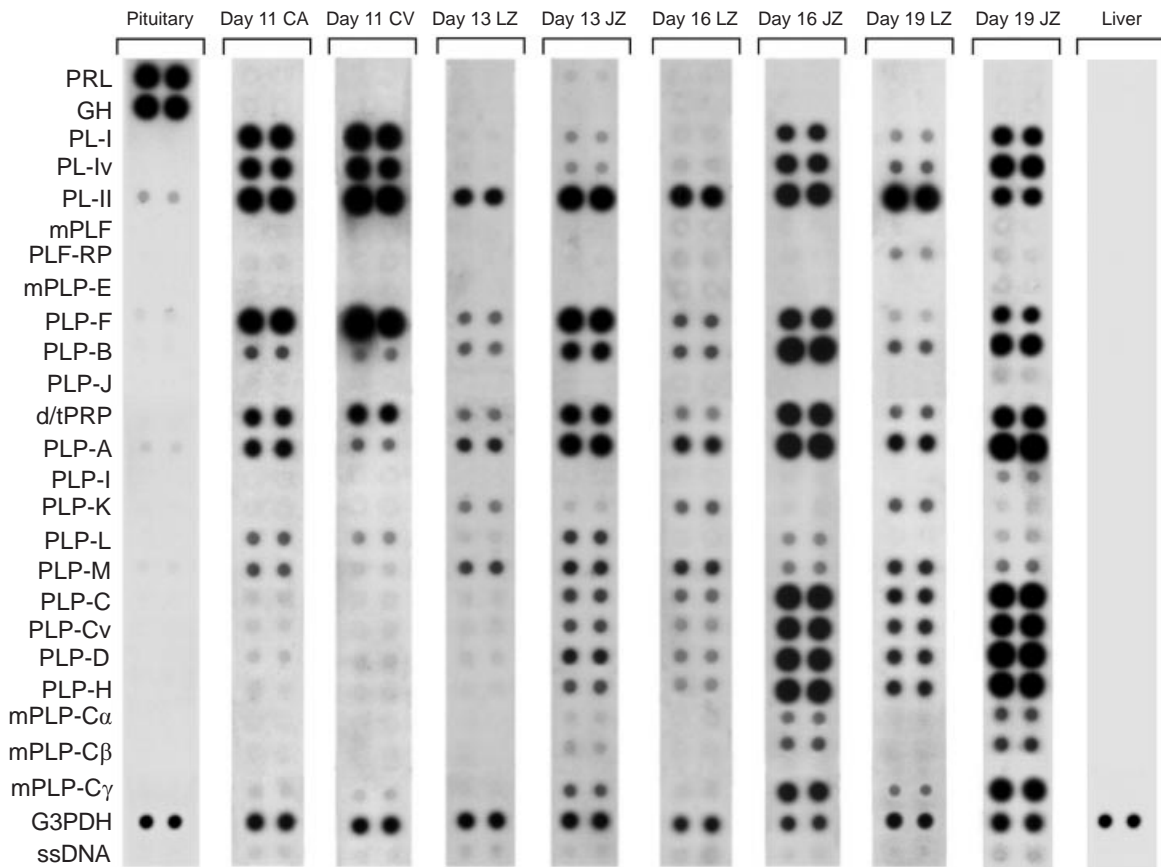
organized in groups of functionally or structurally related prolactin family members (Fig. 2). Some members of the prolactin family exhibited reciprocal patterns of placental expression. PL-I, PLP-A and PLP-E mRNA abundance significantly declined as gestation advanced, whereas PL-II, PLP-F, PLP-C $\alpha$ , PLP-C $\beta$ , PLP-C $\gamma$ , PLP-L and PLP-M mRNA abundance significantly increased as gestation advanced ( $P < 0.01$ ). Other members of the prolactin family exhibited a more dynamic gestational pattern of gene expression. Proliferin, proliferin-RP, PLP-B and PLP-K increased from day 11 to day 13 of gestation and their expression was reduced during the later stages of pregnancy ( $P < 0.01$ ). Prolactin family gene expression was not detected in the mouse liver.



**Fig. 2.** Expression patterns of selected members of the prolactin (PRL) family during pregnancy in mice. Expression was determined by densitometry of PRL family miniarray assays. cDNAs for all members of the mouse PRL gene family and five unique members of the rat PRL gene family (rPLP-C, rPLP-Cv, rPLP-D, rPLP-H, rPLP-I) were isolated, characterized and spotted on to nylon membranes. Total RNA was prepared from mouse anterior pituitary, decidua from day 8 of gestation and placental tissues from day 11, day 13, day 16 and day 19 of gestation, and PRL family expression assessed with the PRL family miniarray assay. Results are organized in groups of functionally or structurally related PRL family members. Data are presented relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression as means  $\pm$  SEM of six replicates. PL, placental lactogen; PLF, proliferin; PLF-RP, proliferin-related protein; PLP, prolactin-like protein.

The prolactin family endocrine phenotype of anterior pituitary, placental and liver tissues of the rat was assessed with the prolactin family miniarray (Fig. 3). Anterior pituitary tissues expressed abundant amounts of prolactin and growth hormone, whereas prolactin family gene expression was not detectable in the rat liver. Rat placental tissues are amenable to additional dissection. Dissected

tissue compartments were analysed. On day 11 of gestation, chorioallantoic and choriovitelline placental tissues exhibited similar expression patterns, except for a reduction in PLP-A, PLP-L and PLP-M mRNA expression in choriovitelline placental tissues. Examination of prolactin family gene expression in chorioallantoic placental compartments revealed some unique features. Proliferin-RP and PLP-K



**Fig. 3.** Evaluation of rat anterior pituitary and placental phenotypes with the prolactin (PRL) family miniarray assay. cDNAs for all members of the rat PRL gene family and five unique members of the mouse PRL family (mPLF, mPLP-E, mPLP-C $\alpha$ , mPLP-C $\beta$ , mPLP-C $\gamma$ ) were isolated, characterized and spotted on to nylon membranes. Total RNA was prepared from rat anterior pituitary and placental tissues from day 11, day 13, day 16 and day 19 of gestation. Day 11 placental tissues were further dissected into chorioallantoic (CA) and choriovitelline (CV) placentae and tissues obtained from day 13, day 16 and day 19 of gestation into labyrinth zone (LZ) and junctional zone (JZ) compartments. Liver served as a control tissue for the procedure. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as an internal positive control. Salmon sperm DNA (ssDNA) was a negative background control for hybridization. Data presented in this figure are representative of at least three different experiments. GH, growth hormone; PL, placental lactogen; PLF, proliferin; PLF-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual-trophoblast prolactin-related protein.

gene expression were restricted to the labyrinth zone, whereas PLP-I and PLP-L mRNAs and rat mRNAs hybridizing with mouse PLP-C $\alpha$  and PLP- $\beta$  predominated in the junctional zone.

#### *Analysis of trophoblast cell phenotypes with the prolactin family miniarray assay*

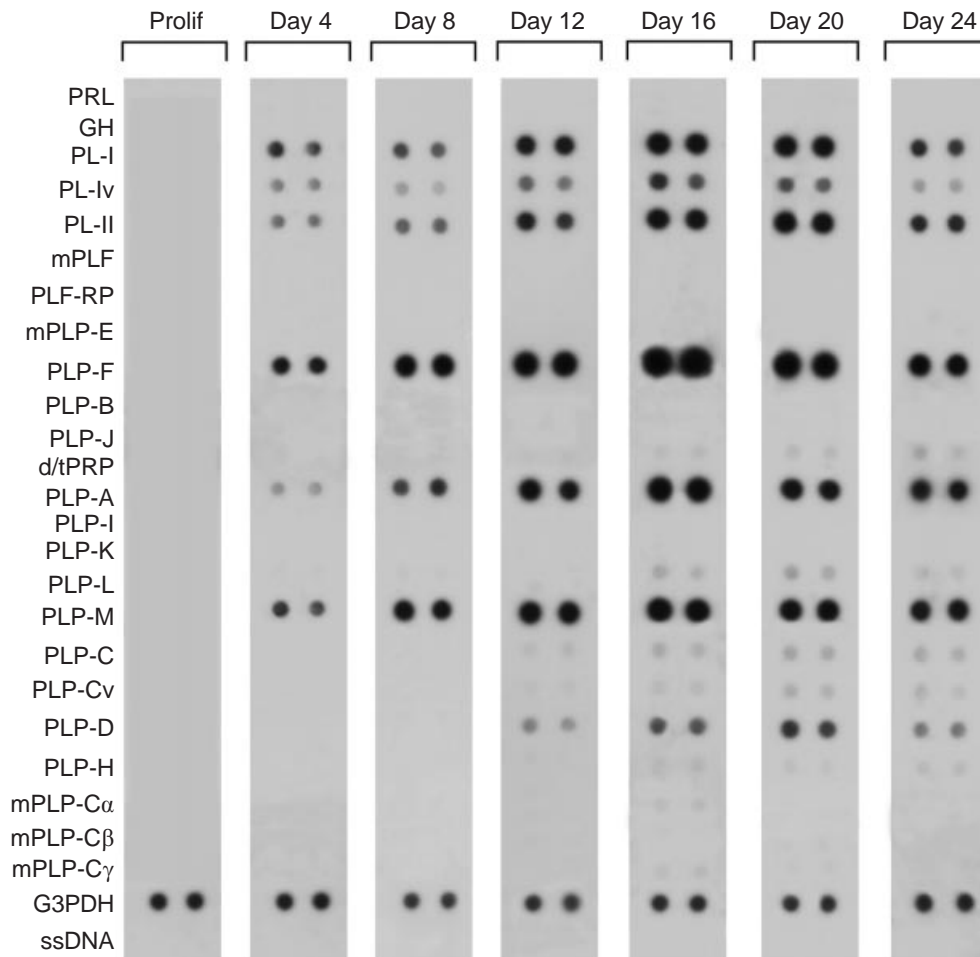
Rcho-1 trophoblast cells can be manipulated to proliferate or differentiate depending upon the culture conditions (Peters *et al.*, 2000). The prolactin family miniarray assay was used to evaluate the prolactin family endocrine phenotype of proliferating and differentiating Rcho-1 trophoblast cells (Figs 4 and 5). PL-I, PL-II, PLP-A, PLP-D, PLP-F and PLP-M showed a progressive and significant increase in expression from the onset of culture until days 16–20 ( $P < 0.01$ ); and each member showed a subsequent significant decrease in expression by day 24 of culture ( $P < 0.01$ ). The pattern of

prolactin family gene expression was consistent with differentiation along the trophoblast giant cell lineage.

The prolactin family miniarray assay was also used to evaluate the prolactin family endocrine phenotype of a mouse trophoblast cell culture model (Fig. 6). Mouse TS cells were examined in proliferating and differentiating cells. Prolactin family gene expression was not detectable in proliferating TS cells. However, after 6 days of culture under differentiating conditions, PL-I, PL-II, proliferin, proliferin-RP, PLP-E and PLP-M mRNAs were detected. The TS cell prolactin family endocrine phenotype was consistent with a trophoblast giant cell mode of differentiation.

## Discussion

The prolactin gene family has undergone an expansion in mice and rats (Soares and Linzer, 2001). Members of the

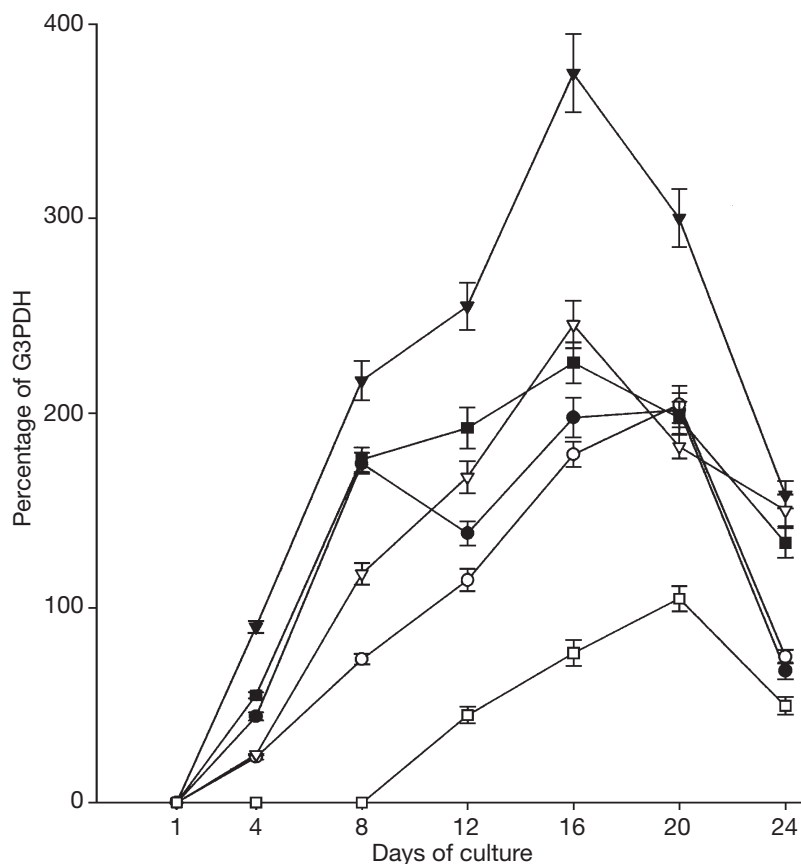


**Fig. 4.** Assessment of rat trophoblast cell phenotypes with the prolactin (PRL) family miniarray assay. Rcho-1 trophoblast cells were analysed under proliferative or differentiating conditions. Proliferating Rcho-1 trophoblast cells were maintained in RPMI-1640 supplemented with 20% fetal bovine serum. Rcho-1 trophoblast cells were induced to differentiate by replacement of the culture medium with NCTC-135 containing 10% horse serum. Cells were harvested under proliferative (Prolif) conditions (day 1 of culture) and on days 4, 8, 12, 16, 20 and 24 of differentiation. Total RNA was isolated and PRL family gene expression assessed with the PRL family miniarray assay. The PRL family miniarray consisted of cDNAs for all members of the rat PRL gene family and five unique members of the mouse PRL family (mPLF, mPLP-E, mPLP-C $\alpha$ , mPLP-C $\beta$ , mPLP-C $\gamma$ ). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as an internal positive control. Salmon sperm DNA (ssDNA) was a negative background control for hybridization. Data presented in this figure are representative of at least three different experiments. GH, growth hormone; PL, placental lactogen; PLF, proliferin; PLF-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual-trophoblast prolactin-related protein.

family are expressed in cell- and temporal-specific patterns (Soares *et al.*, 1991, 1996; Soares and Linzer, 2001). These unique expression patterns were used in the present study to derive a simple array based hybridization assay for evaluating phenotypes of uteroplacental tissues and trophoblast cells.

The prolactin family miniarray assays demonstrate the specificity of expression patterns in the anterior pituitary, uterine decidua and developing chorioallantoic placenta. Tissue- and temporal-specific patterns of gene expression were evident. Prolactin and growth hormone were predominantly expressed in the anterior pituitary; d/tPRP and

PLP-J were abundantly expressed in uterine decidua tissue, and the remaining members of the prolactin family were expressed in dynamic gestational-specific patterns within the developing chorioallantoic placenta. Enrichment for specific placental structures was achieved by mechanical dissection and provided some additional insights into expression profiles. For example, the labyrinthine zone-restricted expression patterns of PLP-K and proliferin-RP (Dai *et al.*, 2000; Sahgal *et al.*, 2000) were verified. Overall expression patterns determined with the prolactin family miniarray assays were similar to those previously reported

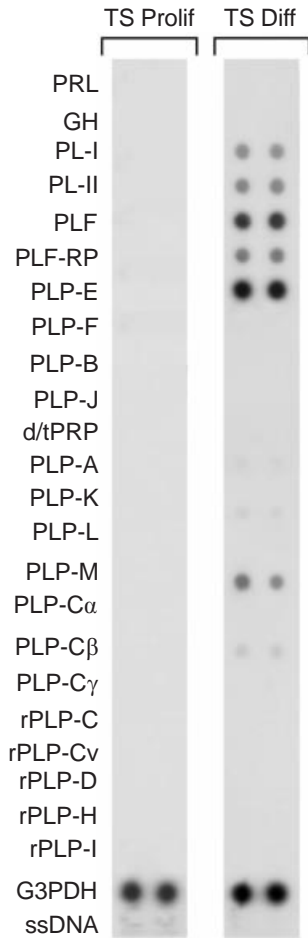


**Fig. 5.** Expression patterns of selected members of the prolactin (PRL) family during rat trophoblast cell differentiation. ●: PL-I; ○: PL-II; ▼: PLP-F; ▽: PLP-A; ■: PLP-M; □: PLP-D. Expression was determined by densitometry of PRL family miniarray assays as shown in Fig. 4. Rcho-1 trophoblast cells were analysed under proliferative or differentiating conditions. Proliferating Rcho-1 trophoblast cells were maintained in RPMI-1640 supplemented with 20% fetal bovine serum. Rcho-1 trophoblast cells were induced to differentiate by replacement of the culture medium with NCTC-135 containing 10% horse serum. Cells were harvested under proliferative conditions (day 1 of culture) and on days 4, 8, 12, 16, 20 and 24 of differentiation. Total RNA was isolated and PRL family gene expression assessed with the PRL family miniarray assay. The PRL family miniarray consisted of cDNAs for all members of the rat PRL gene family and five unique members of the mouse PRL family (mPLF, mPLP-E, mPLP-C $\alpha$ , mPLP-C $\beta$ , mPLP-C $\gamma$ ). Data are presented relative to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) expression as means  $\pm$  SEM of six replicates. PL, placental lactogen; PLP, prolactin-like protein.

for each member of the prolactin family (see Soares *et al.*, 1991, 1996; Soares and Linzer, 2001). The miniarray assay also provided new insights into the relative expression among members of the prolactin family. Some members of the family were abundantly expressed relative to other family members. Relative gene expression was gestationally dependent. The prolactin family miniarray assay should prove to be a useful means of assessing placental phenotypes in normal and genetically mutant mice and rats.

The prolactin family miniarray assay is an excellent means of assessing the differentiation status of trophoblast cells. The chorioallantoic placenta comprises two structurally and functionally distinct regions (Soares *et al.*, 1996):

(i) junctional zone and (ii) labyrinth 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, whereas the labyrinth zone is located adjacent to the developing embryo and contains trophoblast cells, maternal vascular channels and fetal vessels. 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). Each of these types of cell has unique functional and morphological attributes. The trophoblast giant cell is the first differentiated



**Fig. 6.** Assessment of mouse trophoblast stem (TS) cell phenotypes with the prolactin (PRL) family miniarray assay. Proliferating (TS Prolif) and differentiating (TS Diff) cells were evaluated. Proliferating TS cells were maintained in RPMI-1640 supplemented with medium conditioned by mouse embryonic fibroblasts, fibroblast growth factor 4 (FGF-4), heparin and 20% fetal bovine serum. TS cells were induced to differentiate after removal of the conditioned medium, FGF-4 and heparin supplements. Cells were harvested, total RNA isolated and PRL family expression assessed with the PRL family miniarray assay. The PRL family miniarray consisted of cDNAs for all members of the mouse PRL gene family and five unique members of the rat PRL gene family (rPLP-C, rPLP-Cv, rPLP-D, rPLP-H, rPLP-I). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) served as an internal positive control. Salmon sperm DNA (ssDNA) was a negative background control for hybridization. Data presented in this figure are representative of at least three different experiments. GH, growth hormone; PL, placental lactogen; PLF, proliferin; PLF-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual-trophoblast prolactin-related protein.

type of cell to arise during development of the trophoblast lineage. Giant cells possess invasive and endocrine activities and are critically situated at the maternal interface. Spongiotrophoblast cells are morphologically distinct from trophoblast giant cells. However, similar to trophoblast giant cells, spongiotrophoblast cells exhibit endocrine activities, including expression of a subset of members of the prolactin

gene family. Glycogen cells accumulate glycogen and are a potential energy reserve. Syncytial trophoblast cells are multinucleated cells and are implicated in bidirectional transport of nutrients and wastes. Two *in vitro* cell culture models were evaluated in the present study. Rcho-1 trophoblast cells are a rat trophoblast stem cell population that can be manipulated to differentiate (Faria and Soares, 1991; Peters *et al.*, 2000). As Rcho-1 trophoblast cells progressed from a proliferative state to a differentiated state, they exhibited an increased expression of specific members of the prolactin family. PL-I, PL-II, PLP-A, PLP-F and PLP-M mRNAs showed prominent increases in expression as trophoblast cells differentiated. The differentiation-dependent activation of PL-I, PL-II, PLP-A and PLP-F has previously been reported (Faria and Soares, 1991; Sahgal *et al.*, 2000); however, this is the first demonstration that PLP-M mRNA is increased in differentiated trophoblast cells. Expression of other prolactin family members was increased to a lesser extent and later in the differentiation scheme (for example, PLP-L and members of the PLP-C subfamily, d/tPRP, PLP-C, PLP-Cv, PLP-D, and PLP-H). The absence of expression of prolactin, PLP-B, PLP-I, PLP-J, PLP-K and proliferin-RP reinforces the restriction of Rcho-1 trophoblast cell differentiation to the trophoblast giant cell lineage (Faria and Soares, 1991; Peters *et al.*, 2000). Prolactin is expressed in the lactotroph cell lineage and expression is low in decidua (Prigent-Tessier *et al.*, 1999; Dasen and Rosenfeld, 2001); PLP-B is expressed during early decidual cell development and in the spongiotrophoblast cell lineage of the chorioallantoic placenta (Croze *et al.*, 1990; Cohick *et al.*, 1997); PLP-I expression is restricted to late gestation spongiotrophoblast cells (Wiemers *et al.*, in press); PLP-J is expressed in a decidual cell-restricted pattern (Hiraoka *et al.*, 1999; Toft and Linzer, 1999; Dai *et al.*, 2000); and PLP-K and PLF-RP gene activation is limited to labyrinthine trophoblast in the rat chorioallantoic placenta (Dai *et al.*, 2000; Sahgal *et al.*, 2000). The prolactin family miniarray was also used to evaluate the differentiation of mouse TS cells. TS cells are capable of differentiating into each of the trophoblast cell lineages (Tanaka *et al.*, 1998). Prolactin family gene expression was not detectable in proliferating TS cells. However, *in vitro* differentiated TS cells expressed several members of the prolactin family. The phenotype of the differentiated TS cells was consistent with progression along the trophoblast giant cell lineage.

Expression of the prolactin family was not detectable in the liver. An assortment of other normal adult tissues (brain, thymus, heart, lung, stomach, intestine, pancreas, spleen, kidney, adrenal, muscle, adipose, prostate gland, testis, ovary and epididymis) was also surveyed with the prolactin family miniarray assay. These tissues exhibit minimal to non-detectable expression of the prolactin family (data not shown). There is some evidence for the reactivation of proliferin, a member of the prolactin family, in tissues undergoing pathological processes (Fassett *et al.*, 2001; Toft *et al.*, 2001). Such a finding is not surprising since the action of proliferin on endothelial cells during the establishment of

pregnancy may also be relevant to the control of endothelial cells in diseased tissues. Other members of the prolactin family regulate haematopoietic, immune and inflammatory cells (Soares and Linzer, 2001) and by analogy may also be reactivated during the pathogenesis of disease. The prolactin family miniarray may prove to be a useful tool for screening the activation of prolactin family members in pathological tissue specimens.

The prolactin family miniarray assays provided insights about orthologous genes in mice and rats. Mouse and rat orthologues have been identified for most, but not all, members of the prolactin family. In the mouse prolactin family miniarray rat prolactin family paralogues, which are not orthologous to any known mouse prolactin family members, were included. Similarly, in the rat prolactin family miniarray mouse prolactin family paralogues, which are not orthologous to any known rat prolactin family members, were included. In some cases evidence was found for orthologous genes in both species, whereas in other cases there was support for the presence of non-orthologous prolactin family paralogues. Genes orthologous to proliferin and PLP-E do not appear to be expressed in rats. PLP-E and PLP-F are structurally related and represent part of a placental signalling system involved in the regulation of haematopoiesis (Lin and Linzer, 1999; Bittorf *et al.*, 2000). In mice, PLP-E is expressed from early postimplantation stages to midgestation, while PLP-F expression is increased only during the later stages of gestation. In contrast, during pregnancy in rats, PLP-F is increased from early postimplantation stages until the end of pregnancy. Thus, in mice and rats, two slightly different strategies may be used to regulate haematopoiesis during pregnancy (a dual complementary hormone system versus a single hormone system). Additional marked species differences in gestational profiles for PLP-A and PLP-B were also apparent. In mice, expression of both hormones peaks at midgestation, whereas in rats PLP-A and PLP-B expression predominates during the latter half of pregnancy. Hybridization signals were observed for rat placental RNA samples with mouse PLP-C subfamily members. Whether these hybridization signals indicate the presence of rat orthologues or cross-hybridization with rat PLP-C subfamily members remains to be determined. Based on these observations, we propose that the prolactin family miniarray assay may be a useful tool for discovering orthologous genes in other species.

There are some limitations to the prolactin family miniarray assay. The assay is based on nucleotide hybridization and thus discrimination of closely related transcripts is problematic. This is particularly evident for relatives of the PL-I and PLP-C subfamilies. Additionally, the assay does not replace techniques that are characterized by greater specificity (RT-PCR) or techniques that provide precise cellular localization (for example, *in situ* hybridization).

In conclusion, the prolactin family miniarray assay is a technique that provides meaningful information about placental and trophoblast endocrine cell phenotypes and may be useful for other purposes including the identifica-

tion of new tissue sources of prolactin family members and the discovery of orthologous cDNAs in other species.

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