

## Phenotypic Analysis of the Rat Placenta

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### Summary

The rat is an important model for studying the biology of trophoblast-uterine development. This chapter describes methods that are useful for the characterization of the rat uteroplacental compartment.

**Key Words:** Rat; chorioallantoic placenta; choriovitelline placenta; junctional zone; labyrinth zone; metrial gland; spongiotrophoblast; trophoblast giant cells; trophoblast cell invasion; prolactin gene family.

### 1. Introduction

In this chapter, we discuss the rat as an experimental model for investigations directed toward pregnancy and the uteroplacental interface. We present an overview on the organization of the rat maternal–fetal interface and present methods for studying the rat placenta and trophoblast cells.

#### ***1.1. Merits of the Rat as an Animal Model for Placental Research***

Historically, the rat has been a valuable model for studying most aspects of reproduction and, in many areas, still remains the preferred model system. The rat is the dominant preclinical model system used by the pharmaceutical and agro-chemical industries. Genetic approaches for analysis of the rat are rapidly advancing. The first draft of the rat genome is completed (*1*) and significant progress is being made in developing strategies for the genetic manipulation of the rat, including transgenic (*2–7*), in vivo mutagenesis (*8*), and nuclear transfer (*9,10*). Finally, panels of consomic rat strains have been established, which will permit a systematic and physiologically relevant dissection of the rat genome (*11*).

The use of animal model systems provides an essential tool for dissecting molecular mechanisms controlling cellular development. The maternal–fetal interface is no exception. The premise of employing any animal model system is that if the process being studied is fundamental it will likely demonstrate conservation across species. Although there are some differences in the organization of the rodent vs the primate maternal–fetal interface, there are overriding similarities in the functions and lineages of cells comprising the maternal–fetal interface. Among these are events transpiring during the last week of gestation in the rat. During this latter phase of pregnancy in the rat, trophoblast cells exit the chorioallantoic placenta and invade into the uterine endometrium, where they establish intimate relationships with the uterine vasculature (12). These invasive events in the rat are remarkably similar to invasive events occurring during the latter stages of the first trimester and throughout the second trimester of pregnancy in the human (13).

If we can understand and appreciate biological processes at the maternal–fetal interface in species that can be experimentally manipulated (e.g., rat, mouse), then we can more intelligently study the development of the human maternal–fetal interface and identify pivotal junctures of cellular control, facilitating diagnosis and therapeutic intervention.

In some instances, cross-species similarities may prevail, whereas in other cases, the differences may be most compelling. Nonetheless, our appreciation for the biology of pregnancy increases. Animal models provide us with a means of studying biological phenomena at levels that are not ethically permissible with humans. Viviparity is vital to the success of our species. Mechanisms that underlie viviparity will exhibit some level of conservation.

## **1.2. Overview of the Organization of the Uteroplacental Compartment of the Rat**

The uteroplacental compartment of the rat is similar to the mouse and possesses similarities and differences to the organization of the uteroplacental compartment of other species with hemochorial placentation (14–17). This has led to the utilization of an assortment of terms to describe components of the uteroplacental compartment. Schematic representations of the rat uteroplacental compartment are presented in **Figs. 1** and **2**.

The site where blood enters the uterus determines the orientation of the uteroplacental compartment. This region is referred to as the *mesometrial* compartment, and the opposite end is termed the *antimesometrial* compartment. The uterine mesometrial compartment is prominently comprised of stromal cells, blood vessels (endothelial cells, smooth muscle cells), immune/inflammatory cells (natural killer cells, macrophages), smooth muscle cells of the myometrium, and trophoblast cells. Cellular composition of this compartment

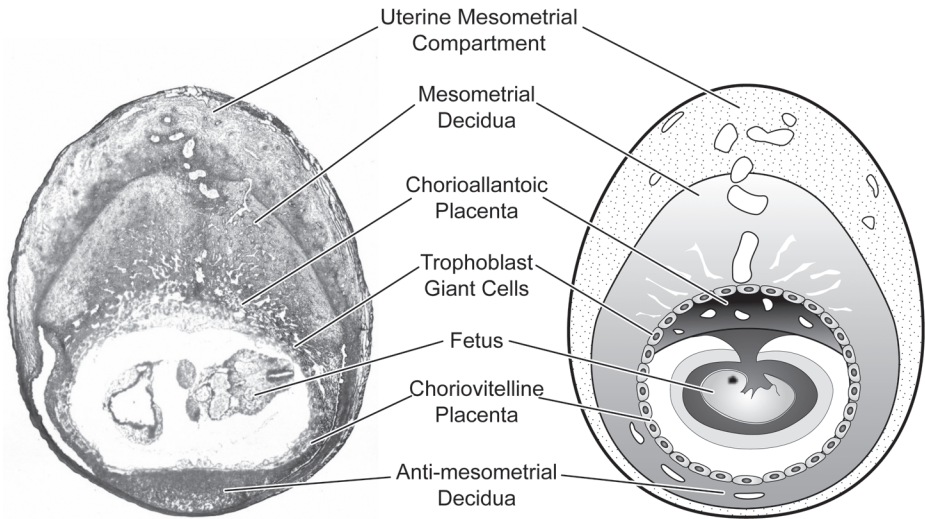


Fig. 1 (*see* companion CD for color version). Hematoxylin and eosin-stained tissue section of the midgestation rat uteroplacental compartment (left panel, day 11 of gestation) and a corresponding schematic diagram (right panel).

is dynamic and has gestation-stage dependent and species-specific characteristics. Following implantation, natural killer cells expand in number, and infiltrate the mesometrial decidua, located adjacent to the developing chorioallantoic placenta. Decidual cells are derived from uterine stromal cells and exhibit functional differences depending upon their location (18,19). Mesometrial decidua is the site of extensive vascular remodeling, whereas antimesometrial decidual cells are conspicuous in their elaboration of cytokines, including members of the prolactin (PRL) family (20). A triangle-shaped area rich in blood vessels is situated between the mesometrial decidua and the surface of the uterus (21,22). This region has been referred to by the terms “mesometrial triangle,” “metrial gland,” and several others (23). As gestation advances, extraembryonic and embryonic structures expand in size and the decidua thins. Accompanying these events, natural killer cells vacate the mesometrial decidua and infiltrate the mesometrial triangle where they associate with the resident vasculature. Subsequently, the antimesometrial deciduum and mesometrial-associated natural killer cells degenerate. As natural killer cells depart, a specialized population of trophoblast cells exits the chorioallantoic placenta and invades into the mesometrial decidua (12). In the mouse, trophoblast invasion is limited to the mesometrial decidua, whereas in the rat, trophoblast cells penetrate through the mesometrial decidua and infiltrate the mesometrial triangle.

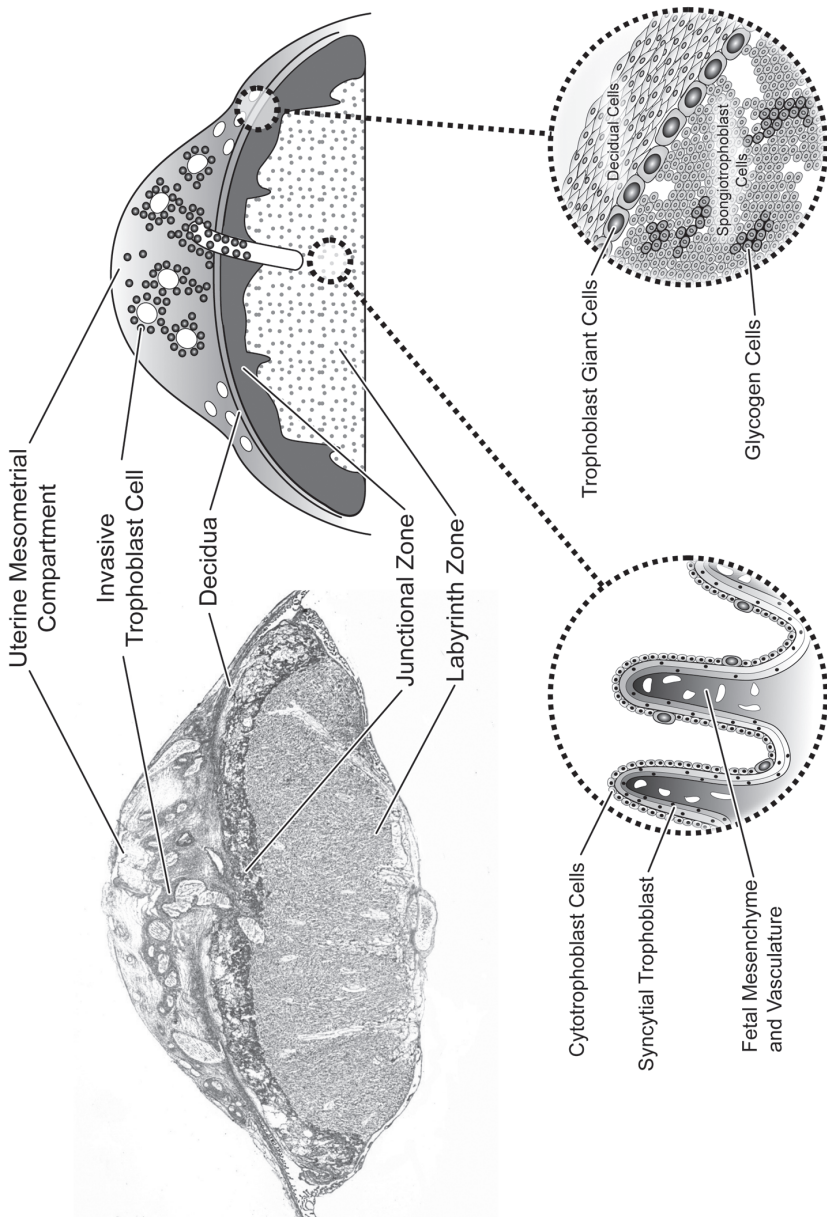


Fig. 2 (see companion CD for color version). Hematoxylin and eosin-stained tissue section of the late gestation rat uteroplacental compartment (left panel, day 18 of gestation) and a corresponding schematic diagram (right panel) with highlighted expanded views of the labyrinth and junctional zones (lower panels).

The chorioallantoic placenta is situated at the base of the mesometrial compartment. It develops from trophoblast stem cells present in the *ectoplacental cone* and generates two recognizable structures: (a) *labyrinth zone* and (b) *junctional zone*. The labyrinth zone arises from the interaction of allantoic mesoderm with the trophoblast stem cell population (24), yielding trophoblast cell syncytialization and establishment of the barrier for maternal–fetal exchange (17). Once the barrier is established, endocrinologically active *trophoblast giant cells* appear within the labyrinth zone. Four trophoblast cell lineages differentiate from trophoblast stem cells within the junctional zone: (a) *trophoblast giant cells*, (b) *spongiotrophoblast cells*, (c) *glycogen cells*, and (d) *invasive trophoblast cells*. Trophoblast giant cells are the first trophoblast cell lineage to develop and, until the last week of gestation, are the most distally located trophoblast cell types within the uterus. Spongiotrophoblast cells are the main constituents of the junctional zone. Trophoblast giant cells, spongiotrophoblast cells, and invasive trophoblast cells are the major endocrine cells of the rat and mouse placenta. Glycogen cells appear during the last week of pregnancy, notably accumulate glycogen, and disappear before the end of pregnancy. The invasive trophoblast cell population first appears at mid-gestation. It consists of trophoblast cells that penetrate and surround the uterine vasculature present within the developing chorioallantoic placenta. As gestation advances, invasive trophoblast cells exit the junctional zone and enter the mesometrial compartment. Three terms have been used to describe the invasive trophoblast cells (25). *Endovascular* trophoblast cells replace the endothelium, *intramural* trophoblast cells are embedded within the vascular wall, and *interstitial* trophoblast cells are situated between the vasculature.

### 1.3. Investigation of the Rat Placenta

In this chapter, we describe methods for (1) mating and gestational staging; (2) detection of pregnancy during early postimplantation stages; (3) dissection of the midgestation uteroplacental compartment; (4) dissection of the chorioallantoic placenta; (5) establishment of spongiotrophoblast cell primary cultures; (6) isolation of the metrial gland; (7) chemical induction of fetal death; and (8) monitoring the phenotype of cells within the uteroplacental compartment. The outlined protocols are based on our experience in working with the rat placenta over the past two decades.

## 2. Materials

### 2.1. Mating and Gestational Staging

1. Holtzman rats are obtained from Harlan Sprague-Dawley (Indianapolis, IN).
2. Saline solution (0.9% NaCl).
3. Glass slide with wells.
4. Microscope (×40–100 magnification).

## **2.2. Detection of Pregnancy During Early Post Implantation Stages**

Chicago Blue B (1% solution; Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH, cat. no. CX685 B364).

## **2.3. Mid-Gestation Placental Dissection**

1. Dissecting microscope ( $\times 10$ – $20$  magnification).
2. Hank's balanced salt solution (HBSS; Sigma Chemical Company, St. Louis, MO, cat. no. H-387).
3. Fine forceps and microdissecting spring scissors (Roboz Surgical Instrument Co., Gaithersburg, MD, cat. nos. RS-5155 and RS-5602, respectively).

## **2.4. Chorioallantoic Placental Dissection**

1. Dissecting microscope ( $\times 10$ – $20$  magnification).
2. HBSS (Sigma).
3. Fine forceps and microdissecting spring scissors (Roboz).
4. 23-gauge needles (BD Biosciences, Franklin Lakes, NJ, cat. no. 305134).

## **2.5. Primary Culture of Spongiotrophoblast Cells**

1. Fine forceps and microdissecting spring scissors (Roboz).
2. Dispase II (Roche Diagnostic Corporation Indianapolis, IN, cat. no. 295825).
3. DNase I (Sigma, cat. no. D4263).
4. HBSS (Sigma).
5. Dulbecco's modified Eagle's medium (DMEM) culture medium (Mediatech Cellgro, Herdon, VA, cat. no. 10-017-CV) supplemented with penicillin and streptomycin (Mediatech Cellgro) and 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA, cat. no. S11150).
6. Nylon mesh cell strainers (70  $\mu\text{m}$ ; BD Biosciences, cat. no. 352350).
7. Percoll (Amersham Biosciences, Uppsala, Sweden, cat. no. 17-089-02).

## **2.6. Metrial Gland Isolation**

1. Fine forceps and microdissecting spring scissors (Roboz).
2. HBSS (Sigma).

## **2.7. Chemical Induction of Fetal Death**

Digoxin (Elkin-Sinn, Cherry Hill, NJ).

## **2.8. Monitoring the Phenotype of Cells Within the Uteroplacental Compartment**

1. TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, cat. no. 15596-018).
2. 1% Formaldehyde-agarose gels. Formaldehyde (Fisher Scientific, Pittsburgh, PA, cat. no. F79-4); agarose (Sigma, cat. no. A-9539).
3. Nylon membranes (Nytran Super Charge, Schleicher & Schuell Biosciences, Inc., Keene, NH, cat. no. 10416296).

4. Crosslinker (Model XL-1000, Spectronics Corporation, Westbury, NY).
5. [ $\alpha$ -P<sup>32</sup>]dCTP (Perkin Elmer, Boston, MA, cat. no. Blu/NEG/0134).
6. Plasmids containing cDNAs for the transcripts of interest (**Table 1**).

**2.8.1. PRL Family DNA Miniarray (32) (see Note 12)**

1. Polymerase chain reaction (PCR)-amplified cDNA for each of the members of PRL family is spotted, in duplicate, onto nylon membranes (Schleicher & Schuell Biosciences, Inc.), crosslinked, and stored at 4°C until used.
2. TRIzol reagent (Invitrogen).
3. [ $\alpha$ P<sup>32</sup>]dCTP (Perkin Elmer, Boston, MA).
4. Micro bio-spin columns (Bio-Rad Laboratories, Hercules, CA, cat. no. 732-6223).
5. Denhardt's reagent (50X Denhardt's reagent): 1% ficoll (Sigma, cat. no. F-4375), 1% polyvinylpyrrolidone (Sigma, cat. no. P-5288), 1% bovine serum albumin (BSA; Fraction V, Fisher, cat. no. BP1600-100) diluted in distilled water.
6. Deionized formamide (Sigma, cat. no. F9037).
7. Salmon sperm DNA (Invitrogen, cat. no. 15632-011).
8. SSPE buffer (20X SSPE): add 175 g sodium chloride, 27.6 g sodium phosphate monobasic, 7.4 g ethylenediamine tetraacetic acid (EDTA) to 1 L of distilled water.
9. 0.1% sodium dodecyl sulfate (SDS; Fisher, cat. no. BP 166-500).
10. Kodak Bio-Max film (Kodak, Rochester, NY, cat. no. 829-4985).

**2.8.2. In Situ Hybridization (33,34) (see Note 13)**

1. Plasmids containing cDNAs for the transcript of interest are used as templates to synthesize sense and anti-sense digoxigenin-labeled riboprobes (**Table 1**).
2. Dry-ice-cooled heptane (Fisher, cat. no. 03008-1).
3. Cryostat (Model No. CM 1850-3-1, Lieca Microsystems, Germany).
4. 4% paraformaldehyde (P-6148, Sigma) in phosphate-buffered saline (PBS) at 4°C.
5. Deionized formamide (Sigma).
6. 1X Denhardt's reagent.
7. 10% Dextran sulfate (Fisher, cat. no. BP 1585-100).
8. Salmon sperm DNA (Invitrogen).
9. Standard saline citrate (SSC; 20X SSC): 175.3 g sodium chloride, 88.2 g sodium citrate in 1 L of distilled water.
10. RNase-A (Sigma, cat. no. R-6513).
11. Dig-High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostic Corporation, cat. no. 1585614).

**2.9. Monitoring Protein Expression**

**2.9.1. Western Blotting**

1. Reagents for polyacrylamide gel electrophoresis and electrophoretic transfer (BioRad Laboratories).

**Table 1**  
**cDNAs Used in the Phenotypic Analysis of Cells Within the Uteroplacental Compartment**

Cell type	Gene	Gestation stage	GenBank accession no.	Reference
<i>Trophoblast</i>				
Ectoplacental cone	PLF-RP	Early	NM_053364	37
Trophoblast giant cell	PL-I	Early to mid	D21103	31
	PL-II	Mid to late	M13749	31,38
	PLP-F $\alpha$	Early to mid	NM_022530	37, unpublished <sup>a</sup>
	P450scc	Early to late	J05156	39,40
	P450c17	Mid to late	NM_012753	40,41
Spongiotrophoblast	3 $\beta$ HSD	Early to late	L17138	Unpublished <sup>b</sup>
	PLP-B	Mid to late	M31155	42,43
	PLP-F $\beta$	Mid to late	AY741310	Unpublished <sup>a</sup>
	SSP	Mid to late	NM_172073	44
Labyrinthine TGC	PL-II	Mid to late	M13749	42,45
	PLP-K	Mid to late	NM_138861	32
	PLF-RP	Mid to late	NM_053364	37
Syncytial trophoblast	FABP3	Mid to late	NM_024162	46
	Alk Phos	Mid to late	NM_013059	47
Invasive trophoblast	PLP-L	Mid to late	NM_138527	12
	PLP-M	Mid to late	NM_053791	12
	PLP-N	Mid to late	NM_153738	48
	IGF-II	Mid to late	X17012	49, unpublished <sup>c</sup>
<i>Decidual cells</i>				
Mesometrial	$\alpha$ 2-MG	Early to mid	NM_012488	50
Antimesometrial	dPRP	Early to mid	NM_022846	51,52
	PLP-J	Early to mid	NM_031316	32
<i>Natural killer cells</i>	Osteopontin	Mid	NM_012881	Unpublished <sup>d</sup>

Abbreviations: TGC, Trophoblast giant cell; PLF-RP, proliferin-related protein; PL, placental lactogen, PLP, prolactin-like protein; P450scc, side chain cleavage; P450c17, 17 $\alpha$  hydroxylase; 3 $\beta$ HSD, 3 $\beta$  hydroxysteroid dehydrogenase; SSP, spongiotrophoblast-specific protein; FABP3, fatty acid binding protein-3; IGF-II, insulin-like growth factor-II;  $\alpha$ 2-MG,  $\alpha$ 2-macroglobulin; dPRP, decidual prolactin-related protein.

<sup>a</sup>Ho-Chen, J., Bustamante, J. J., and Soares, M. J., unpublished results.

<sup>b</sup>Canham, L. N. and Soares, M. J., unpublished results.

<sup>c</sup>Ain, R. and Soares, M.J., unpublished results.

<sup>d</sup>Liu, B. and Soares, M.J., unpublished results.

**Table 2**  
**Antibodies Used in the Phenotypic Analysis of Cells Within the Uteroplacental Compartment**

Cell type	Antigen	Gestation stage	Source (Cat. No.)	Reference
<i>Trophoblast</i>	Cytokeratin	All stages	Sigma Chemical Co., St. Louis, MO (C2931)	<b>12</b>
Trophoblast giant cell	PL-I	Early to mid	Chemicon International, Temecula, CA (AB1288)	<b>31,53</b>
	PL-II	Mid to late	Chemicon (AB1289)	<b>31,38,54</b>
	PLP-A	Mid to late	Chemicon (AB1290)	<b>55,56</b>
	P450scc	Early to late	Chemicon (AB1244, AB1294)	<b>39,57</b>
Spongiotrophoblast	P450c17	Mid to late	<i>see refs.</i>	<b>40,58</b>
	PLP-B	Mid to late	Chemicon (AB1291)	<b>43</b>
Labyrinthine TGC	SSP	Mid to late	<i>see ref.</i>	<b>44</b>
	PL-II	Mid to late	Chemicon (AB1289)	<b>38</b>
Syncytial trophoblast	FABP3	Mid to late	<i>see ref.</i>	<b>46</b>
	Alk Phos	Mid to late	<i>see ref.</i>	<b>47</b>
<i>Decidual cells</i>				
Mesometrial	$\alpha$ 2-MG	Early to mid	<i>see ref.</i>	<b>50</b>
Antimesometrial	dPRP	Early to mid	Chemicon (AB1293)	<b>52,59</b>
	PLP-J	Early to mid	<i>see ref.</i>	Unpublished <sup>a</sup>
<i>Natural killer cells</i>	Perforin	Mid	Torrey Pines Biolabs, Houston, TX (TP251)	<b>12</b>
<i>Macrophages</i>	ED-1/ED-2	All stages	Serotec Inc., Raleigh, NC (MCA341R/ MCA342R)	Unpublished <sup>b</sup>
<i>Smooth muscle cells</i>	Sm actin	All stages	Sigma (A2547)	Unpublished <sup>b</sup>

Abbreviations: TGC, trophoblast giant cells; PL, placental lactogen, PLP, prolactin-like protein; P450scc, side chain cleavage; P450c17, 17 $\alpha$  hydroxylase; SSP, spongiotrophoblast-specific protein; FABP3, fatty acid binding protein-3;  $\beta$ 2-MG,  $\alpha$ 2-macroglobulin; dPRP, decidual prolactin-related protein; Sm, smooth muscle.

<sup>a</sup>Alam, S. M. K., Konno, T., and Soares, M. J., unpublished results.

<sup>b</sup>Ain, R. and Soares, M. J., unpublished results.

2. Nitrocellulose (Optitran, Schleicher & Schuell Biosciences, Inc., Cat. No. BA-S 85).
3. Antibodies to the protein of interest (**Table 2**).

### 2.9.2. Immunocytochemistry

1. Dry-ice-cooled heptane (Fisher).
2. Cryostat (Leica).
3. Antibodies to the antigen(s) of interest (**Table 2**).

### 3. Methods

#### 3.1. Mating and Gestational Staging

1. The rats are maintained on a 14 h light:10 h dark lighting schedule with lights on at 0600 h (*see Note 1*).
2. Adult males, preferably older than 10 wk of age, are placed one per cage.
3. Adult females, generally 7–10 wk of age, are transferred to a cage with a male (no more than two females per male). The fur on one of the females is generally marked with a dye to distinguish it from the other female.
4. Every morning between 0800 and 0900 h, each female cohabiting a cage with a male, is removed from the cage for the purpose of obtaining a vaginal lavage.
5. A few hundred microliters of saline are delivered with a pipette into the vagina of the female and recovered with the same pipet.
6. The contents of each saline lavage are transferred to a well within a multi-well glass plate.
7. After all of the vaginal lavages are collected, they are examined with the aid of a microscope ( $\times 40$ – $100$  magnification).
8. The presence of sperm in the lavage is recorded, as is the cellular content of the lavage (*see Notes 2 and 3*).
9. The sperm positive females are transferred to separate cages. The presence of sperm in the vaginal lavage is considered day 0 of pregnancy (*see Note 4*).

#### 3.2. Detection of Pregnancy During Early Post Implantation Stages (26) (*see Note 5*)

1. Pregnancy detection within the first 48 h post implantation requires intravenous injection of a vital blue dye, such as Chicago Blue B.
2. A volume of 0.25–0.5 mL/100 g body weight of a 1.0% solution of Chicago Blue B can be injected into the tail vein of the rat.
3. Implantation reactions are identified by the accumulation of blue bands within the uterus after 15 min.

#### 3.3. Mid-Gestation Placental Dissection (27) (*see Note 6*)

1. Embryos with their encapsulating decidual tissues (conceptuses) are retrieved from the uterus from days 10–13 of gestation.
2. Conceptuses are dissected with the aid of a dissecting microscope ( $\times 10$ – $20$  magnification).
3. Tissues are collected and washed with HBSS.
4. The overlying decidua basalis and decidua capsularis are removed with fine forceps and gentle dissection.
5. A cut through the mural pole of the trophoblast layer is made and the trophoblast retracted. Be careful not to cut through the yolk sac/amnion.
6. The visceral yolk, amnion, and embryo are separated from the developing chorioallantoic placenta by cutting at the insertion site of the allantois with microdissection spring scissors.
7. The entire trophoblast component is flattened (allantoic insertion site is up) and can be further separated into chorioallantoic and choriovitelline layers with the

aid of microdissection spring scissors. The inner dark circle of tissue (more vascular) comprising the chorioallantoic tissue is cut away from the lighter surrounding tissue (less vascular) consisting of the choriovitelline tissue.

8. Dissected decidua basalis, decidua capsularis, and trophoblast components can each be processed as required, and/or stored at  $-80^{\circ}\text{C}$ , until further use.

### **3.4. Chorioallantoic Placental Dissection (28) (see Note 7)**

1. Embryos with their encapsulating decidual tissues (conceptuses) can be retrieved from the uterus on days 13 to 21 of gestation.
2. Conceptuses are dissected with the aid of a dissecting microscope ( $\times 10$ – $20$  magnification).
3. The tissues are collected into and washed with HBSS.
4. The overlying decidual basalis tissue and underlying yolk sac/umbilical insertion are removed with fine forceps and microdissection spring scissors.
5. The junctional zone is identified by its pale appearance, due to the absence of fetal blood, and separated from the labyrinth zone, a richly vascularized tissue, with fine forceps and 23-gauge needles.
6. Recovered tissues are rinsed in HBSS, processed as required, and/or stored at  $-80^{\circ}\text{C}$ , until further use.

### **3.5. Primary Culture of Spongiotrophoblast Cells (29) (see Note 8)**

1. Junctional zones from day-13 rat chorioallantoic placentas are dissected under sterile conditions (*see Subheading 3.4.*).
2. Tissues are cut into small pieces with microdissection spring scissors and dissociated with Dispase II (4.8 mg/mL) and DNase I for 1 h at  $37^{\circ}\text{C}$  with continuous shaking.
3. At the end of the digestion, the suspension of cells and tissue fragments are mixed several times with the aid of a Pasteur pipet, and centrifuged.
4. The harvested cells are then resuspended in DMEM culture medium supplemented with 10% FBS and filtered through a nylon mesh (70  $\mu\text{m}$ ).
5. The cell suspension is then centrifuged through a 60% cushion of Percoll for 15 min at room temperature.
6. Cells at the interface are collected, washed with DMEM supplemented with 10% FBS, and cultured in the same medium.
7. The cells can be maintained in DMEM medium containing FBS (1–10%) for 7–10 d.

### **3.6. Metrial Gland Isolation (see Note 9)**

The metrial gland can be isolated from midgestation onward (**Fig. 1**).

1. The uterus is removed from the female rat.
2. Fat and mesenteries are removed from the uterus.
3. Microdissection spring scissors are used to cut each uterine horn along its antimesometrial surface.

4. The decidual–placental–embryo units are removed by gentle dissection from the opened uterus with fine curved forceps.
5. The attachment site of the mesometrial decidua with the uterine wall can be readily identified by its pale appearance and defines the limits of the metrial gland.
6. The wall of the uterus associated with the mesometrial attachment site is removed with microdissection spring scissors and processed as required.

### **3.7. Chemical Induction of Fetal Death (30) (see Note 10)**

1. Pregnant female rats (days 10–13 of gestation) are anesthetized.
2. A midline abdominal incision is made to expose the uterine horns.
3. Digoxin, a cardiac glycoside, is injected into each amniotic cavity (12.5 µg/50 µL in a saline vehicle) through the antimesometrial aspect of the uterus.
4. The abdominal muscle is sutured and the skin is secured with wound clips.

### **3.8. Monitoring the Phenotype of Cells Within the Uteroplacental Compartment**

Several methods can be used to assess the phenotype of the rat uteroplacental compartment, including assessment of mRNA and protein expression.

#### **3.8.1. Monitoring mRNA Expression**

We routinely use northern blotting, the PRL family DNA miniarray, and *in situ* hybridization for monitoring the phenotypes of cells within the rat uteroplacental compartment.

##### **3.8.1.1. NORTHERN BLOTTING (31)**

1. Total RNA is extracted from tissues using TRIzol reagent, resolved in 1% formaldehyde-agarose gels, transferred to nylon membranes, and crosslinked.
2. Blots are probed with  $\alpha$ -<sup>32</sup>P-labeled cDNAs.
3. Glyceraldehyde-3'-phosphate dehydrogenase (G3PDH) cDNA is used to evaluate the integrity and equal loading of RNA samples (see Note 11).

##### **3.8.1.2. PRL FAMILY DNA MINIARRAY (32) (SEE NOTE 12)**

1. Twenty ng of PCR-amplified cDNA for each of the members of PRL family is spotted, in duplicate, onto nylon membranes, crosslinked, and stored at 4°C until used.
2. Total RNA is extracted from tissues using TRIzol reagent.
3. [ $\alpha$ -<sup>32</sup>P]dCTP labeled cDNA probes are generated by reverse transcription using 5 µg of total RNA.
4. Probes are purified using micro bio-spin columns.
5. Membrane filters are briefly rinsed with water and prehybridized for 2 h at 42°C with 5X SSPE containing 5X Denhardt's reagent, 50% deionized formamide, 1% SDS, and salmon sperm DNA (100 µg/mL).
6. Hybridizations are performed overnight with the labeled probes at 42°C.

7. Membranes are washed once with 2X SSPE and 0.1% SDS for 30 min at 42°C and twice with 0.1X SSPE and 0.5% SDS at 60°C for 30 min each.
8. Membranes are then wrapped with plastic wrap and exposed to Kodak Bio-Max film for 1–4 h and developed.

### 3.8.1.3. IN SITU HYBRIDIZATION (33,34) (SEE NOTE 13)

1. Ten-micron cryosections of tissues are prepared and stored at –80°C until used.
2. Plasmids containing cDNAs for the transcript of interest are used as templates to synthesize sense and anti-sense digoxigenin-labeled riboprobes.
3. Tissue sections are air dried and fixed in ice cold 4% paraformaldehyde in PBS for 15 min.
4. Prehybridization is carried out in a humidified chamber at 50°C in 5X SSC, 50% deionized formamide, 1X Denhardt's reagent, 10% dextran sulfate and salmon sperm DNA (100 µg/mL).
5. Hybridizations are performed in the same incubation conditions overnight.
6. Slides are washed in 2X SSC at room temperature for 30 min followed by treatment with RNase-A (100 ng/mL) and additional washes with 2X SSC for 30 min at room temperature, with 2X SSC for 1 h at 65°C, with 0.1X SSC for 1 h at 65°C.
7. Tissue samples are then blocked for 30 min and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500) in blocking buffer for 2 h at room temperature.
8. Slides are then washed and detection is performed using nitro blue tetrazolium (250 µg/mL) and 5-bromo-4-chloro-3-indolyl-phosphate (225 µg/mL).

### 3.8.2. Monitoring Protein Expression

Antibodies can be used to monitor specific protein expression by Western blotting or used to localize the expression of a specific protein/antigen to a cell type within the uteroplacental compartment. **Table 2** contains a list of antibodies useful in the analysis of the rat uteroplacental compartment.

1. Western blotting. Uteroplacental tissues are isolated as described above and extracted consistent with the protein being investigated. Extracts are separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with antibodies specific to the protein of interest.
2. Immunocytochemistry. The intact uteroplacental unit is isolated, frozen in dry-ice-cooled heptane, and stored at –80°C. Cryosections are prepared at 8–10 µm, adhered to glass slides, and probed with antibodies specific to the antigen of interest (*see Note 14*).

## 4. Notes

1. The Holtzman rat is an outbred strain closely related to the Sprague-Dawley rat. The rats are easy to handle. Under the 14 h light:10 h dark lighting schedule the female rats tend to have a 5-d estrous cycle. Our approach for mating and the

pregnancy dating system also applies to other strains. Males used for breeding are obtained at 10 wk of age and usually continue to be effective breeders until approx 1 yr of age. Although, most vendors provide timed-pregnant female rats, we have not found their dating of pregnancies to be reliable and prefer to generate our own timed pregnancies.

2. The process is repeated daily until mating is confirmed by the presence of sperm. Occasionally, seminal plugs are present in the vagina. Unlike the mouse, seminal plugs tend to fall out of the rat vagina. Special suspended caging, if permitted, can be used and dark paper placed in the trays beneath the cages each evening and checked the following morning for the presence of the whitish-yellow, opaque seminal plugs. This is generally the procedure used for generating pseudopregnant rats with vasectomized males.
3. Inspection of the cellular content is helpful. Based on the cellular composition of the vaginal lavage it is possible to determine whether the animal is cycling. Cells present in the vaginal lavage are impacted by circulating concentrations of the ovarian steroid hormones, estrogen and progesterone. Vaginal lavage's containing nucleated and/or nucleated with cornified cells characterize the estrous cycle stage coinciding with behavioral estrus and receptivity. Cornified cells are characteristic at the time of ovulation and lavages containing leukocytes are dominant post-ovulation during the luteal phase. A cycling female cohabiting a cage with a male would raise the question of the male's fertility.
4. It is always important to determine the pregnancy dating system used when reviewing any experimentation with pregnant rats. For us, classifying the presence of vaginal sperm as day 0 of pregnancy is historical. Others consider the presence of sperm in the vagina as day 1 of pregnancy. It would likely be most accurate to consider 1200 h on the day sperm is found to be day 0.5 of pregnancy. It is critical to appreciate that trophoblast/placental development can differ markedly within a day. Thus, regardless, of the pregnancy dating system used, it is imperative to provide the information in any scientific report forthcoming from the research. De Rijk and colleagues have published a useful guide for assessing placental morphology and pregnancy-dependent maternal hematological indices (35).
5. The detection of early postimplantation pregnancy by intravenous injection of a vital dye is based on capillary permeability changes. The vital dyes bind to circulating proteins such as albumin. As the capillary permeability increases after implantation, the circulating dye-bound albumin exits the capillaries and concentrates in the surrounding tissue.
6. The dissection of the midgestation placenta requires practice. In order to maintain the appropriate orientation of maternal, extraembryonic, and embryonic tissues, it is best not to disrupt the amniotic contents. It is also essential to identify mesometrial vs antimesometrial poles. The mesometrial decidua is thicker than the antimesometrial decidua. The shape of the mid-gestation conceptus can be compared to an ice-cream cone (more evident on days 10–11 of gestation). Using such an analogy, the mesometrial pole would be the location of the ice cream. We

have used chorioallantoic and choriovitelline to discriminate between the polar and mural trophoblast components of the midgestation conceptus. Some have argued that the relationship between the mural trophoblast and the yolk sac does not constitute a true choriovitelline or yolk sac placenta (36).

7. Prior to day 13 of gestation, it is difficult to separate the junctional and labyrinth zones. After day 15, it is difficult to completely remove the thinning decidua basalis, which becomes firmly adherent to the junctional zone. Beginning on day 15 of gestation, it also becomes possible to dissect the chorioallantoic zones with fine forceps; needles are not required. Separation of junctional and labyrinth zones is not readily achieved in the mouse and hamster as a result of extensive interdigitation of the zones. It is important to appreciate that even in the rat, the dissection of the junctional and labyrinth zones is not perfect and usually, a small amount of residual contamination is evident.
8. Dissection of the junctional zone from tissue obtained earlier than day 13 of gestation is more time-consuming and yields a nominal amount of starting tissue for the enzymatic dissociation. Junctional zones from later in gestation are easy to dissect but do not yield a good starting cell population. In a limited series of experiments, we have found that the establishment of junctional zone cultures from placentas obtained later during gestation is difficult and the cells show poor viability. The rat day-13 junctional zone cell cultures show no evidence of proliferation of any cell type. These cultures also show modest contamination of vimentin-positive cells (2–3%). The rat day-13 junctional zone cells in primary culture spontaneously differentiate as demonstrated by their expression of members of the placental PRL gene family (29). Most of the cells exhibit a spongiotrophoblast cell phenotype; however, some trophoblast giant cells and syncytial trophoblast cells are also detected.
9. The metrial gland is a heterogeneous tissue. Its landmarks are not well defined. Thus consistency in dissection is a necessity.
10. Digoxin is an effective tool for inducing fetal death at midgestation but does not work well as gestation advances. We have had some success using intra-amniotic injections of potassium chloride to kill fetuses from later in gestation.
11. Interpretation of RNA measurements in the uterus or placenta is directly dependent upon the quality of tissue dissection. Simply labeling a sample containing some placental tissue as placenta does not make it placenta. Most placental samples contain varying amounts of decidual tissue and yolk sac. We have utilized an assortment of different housekeeping genes to monitor RNA integrity and loading efficiency. These have included  $\beta$ -actin, G3PDH,  $\beta$ -tubulin, and 28S ribosomal RNA.
12. The PRL family miniarray assay represents an effective screening tool for monitoring trophoblast cell development. Information can be retrieved regarding decidual and trophoblast cell lineages and temporal aspects of differentiation. The assay is most effective when complementary procedures are employed for monitoring changes in gene expression.

13. In our hands, *in situ* hybridization is a reliable method for identifying cell types contributing to the expression of a specific gene within the uteroplacental compartment. An appreciation of the dynamic changes in uteroplacental morphology is essential to maximize the benefit of the approach.
14. Antibodies are effective tools for identifying and localizing proteins. Each antibody-antigen interaction needs to be optimized for the specific technique employed.

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