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Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: novel endocrine phenotype and regulation

Rupasri Ain, Lindsey N. Canham, and Michael J. Soares*

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

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Abstract

Trophoblast cell invasion into the uterine wall is characteristic of hemochorial placentation. In this report, we examine trophoblast cell invasion in the rat and mouse, the endocrine phenotype of invasive trophoblast cells, and aspects of the regulation of trophoblast cell invasion. In the rat, trophoblast cells exhibit extensive interstitial and endovascular invasion. Trophoblast cells penetrate through the decidua and well into the metrial gland, where they form intimate associations with the vasculature. Trophoblast cell invasion in the mouse is primarily interstitial and is restricted to the mesometrial decidua. Both interstitial and endovascular rat trophoblast cells synthesize a unique set of prolactin (PRL)-like hormones/cytokines, PRL-like protein-A (PLP-A), PLP-L, and PLP-M. Invading mouse trophoblast cells also possess endocrine activities, including the expression of PLP-M and PLP-N. The trafficking of natural killer (NK) cells and trophoblast cells within the mesometrial uterus is reciprocal in both the rat and mouse. As NK cells disappear from the mesometrial compartment, a subpopulation of trophoblast cells exit the chorioallantoic placenta and enter the decidua. Furthermore, the onset of interstitial trophoblast cell invasion is accelerated in mice with a genetic deficiency of NK cells, Tgε26 mice, implicating a possible regulatory role of NK cells in trophoblast cell invasion. Additionally, the NK cell product, interferon-γ (IFNγ), inhibits trophoblast cell outgrowth, and trophoblast cell invasion is accelerated in mice with a genetic deficiency in the IFNγ or the IFNγ receptor. In summary, trophoblast cells invade the uterine wall during the last week of gestation in the rat and mouse and possess a unique endocrine phenotype, and factors present in the uterine mesometrial compartment modulate their invasive behavior.

Keywords: Trophoblast cell invasion; Prolactin-like proteins; Natural killer cells; Interferon-γ; Metrial gland; Placenta; Pregnancy

Introduction

Trophoblast cells have the capacity to differentiate along a multilineage pathway and either organize themselves within the placenta or exit the placenta and colonize the uterine wall. Over the past several years, insights into phenotypes for various rodent intraplacental trophoblast cell lineages have been presented (Soares et al., 1996, 1998; Hemberger and Cross, 2001; Georgiades et al., 2002). Two placental regions can be recognized: (1) junctional zone and (2) labyrinth zone. The junctional zone is comprised of stem cells and three differentiated cell types: (1) trophoblast giant cells, (2) spongiotrophoblast cells, and (3) glycogen cells. Trophoblast giant cells arise by endoreduplication, are situated at the maternal–placental interface, and are one of the major endocrine cells of the placenta (Soares et al., 1996). They synthesize and secrete steroid and peptide hormones. Spongiotrophoblast cells are located immediately beneath the trophoblast giant cell layer and synthesize and secrete peptide hormones. Glycogen cells are embedded among the spongiotrophoblast cells. Beyond their accumulation of glycogen, their biology is not well understood. The labyrinth zone is located at the fetal interface and is comprised of stem cells capable of differentiating into trophoblast giant cells or fusing to form syncytial trophoblast cells. The labyrinthine trophoblast giant cells possess a restricted capacity for hormone production (Soares et al., 1996), whereas syncytial trophoblast cells mediate the transfer of

* Corresponding author. Fax: +1-913-588-8287.
E-mail address: msoares@kumc.edu (M.J. Soares).

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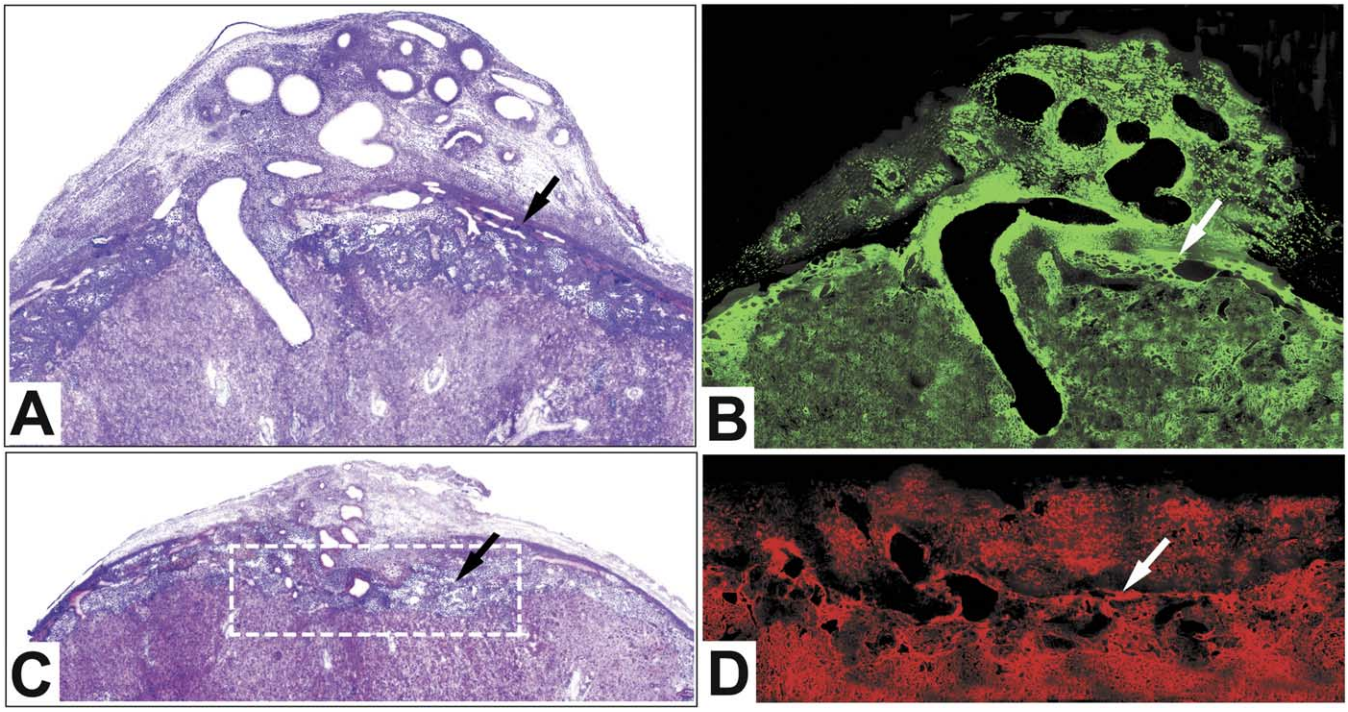


Fig. 1. Identification of invasive trophoblast cells in the rat and mouse. Rat and mouse implantation sites were isolated at day 18 of gestation, and 10-micron cryosections were prepared. Trophoblast cells were identified by cytokeratin immunostaining. (A) Hematoxylin and eosin staining of the rat implantation site. (B) Cytokeratin immunolocalization within the rat implantation site. (C) Hematoxylin and eosin staining of the mouse implantation site. The area shown in the box is presented in (D). (D) Cytokeratin immunolocalization within the mouse implantation site. All magnifications are at 100×. Arrows indicate the trophoblast giant cell boundary between the placenta and decidua.

nutrients and wastes between maternal and fetal compartments (Knipp et al., 1999). The composition of the trophoblastic elements within each zone changes through pregnancy.

Trophoblast giant cells and spongiotrophoblast cells are endocrine cells of the placenta. Among the hormones they produce are a large group of hormones/cytokines belonging to the prolactin (PRL) family (Soares et al., 1998; Soares and Linzer, 2001). The PRL family consists of at least 19 genes in the rat and 26 genes in the mouse (Soares and Linzer, 2001; Wiemers et al, 2003). Both trophoblast cell type and location influence profiles of PRL family gene expression. A variety of biological targets for members of the PRL family have been identified, including the reproductive tract, liver, endothelial cells, immune cells, and inflammatory cells (Soares and Linzer, 2001).

Cells of the trophoblast lineage also exit the placenta and invade the uterine wall. These migratory trophoblast cells establish relationships with the uterine vasculature (Pijnenborg et al., 1981; Enders and Welsh, 1993; Adamson et al., 2002), which is a hallmark of hemochorial placentation (Pijnenborg et al., 1981; Fisher and Damsky, 1993). Two populations of invasive trophoblast cells have been identified: (1) interstitial and (2) endovascular. Interstitial trophoblast cells penetrate through the uterine stroma and are often situated in perivascular locations, whereas endovascular trophoblast cells enter uterine blood vessels, where they can replace endothelial cells (Pijnenborg et al., 1981). The proximity of the trophoblast cells to the uterine vasculature suggests a functional relationship. Aberrant trophoblast cell regulation can result in shallow or the absence of invasion and clinically can be manifested in pregnancy-associated disorders, such as preeclampsia and intrauterine growth restriction (Kingdom, 1999; Goldman-Wohl and Yagel, 2002).

Trophoblast cell invasion has received limited attention in rodents. There is evidence for endovascular and interstitial trophoblast invasion in the rat (Bridgman, 1949; Correia-da-Silva et al., 1999; Vercruysse et al., 2001), hamster (Orsini, 1954), and mouse (Redline and Lu, 1989; Teesalu et al., 1998; Adamson et al., 2002). Invasion is targeted toward the mesometrial triangle, a rich vascular site within the uterus, which ultimately supplies nutrients for the placenta and fetus. Selye and McKeown (1935) used the term “metrial gland” to describe this region of the rat uterus. Signals controlling the entry and function of the migratory trophoblast are not well understood. Among the potential regulators of trophoblast invasion are natural killer (NK) cells, which are prominent constituents of the metrial gland (Head, 1996; Croy et al. 1996). The endocrine phenotype of the migratory trophoblast cells is also not known.

In this report, we have investigated: (1) trophoblast invasion in rats and mice, (2) the endocrine phenotype of these invading trophoblast cells, and (3) the potential mod-

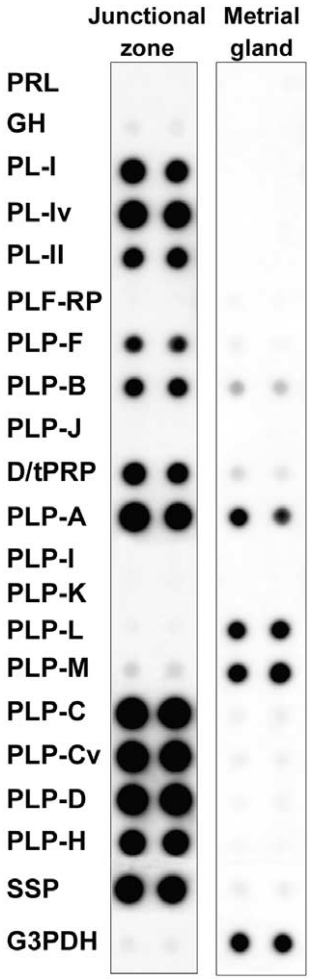


Fig. 2. Expression of members of the PRL gene family in the rat placenta and metrial gland. PRL family miniarray analyses of RNAs isolated from the junctional zone of the chorioallantoic placenta and the metrial gland. Total RNA samples were isolated from junctional zone and metrial gland tissues on day 18 of gestation, radiolabeled by reverse-transcription, and used as hybridization probes for rat PRL family miniarrays. Spongiotrophoblast-specific protein (SSP) and glyceraldehyde-3′-phosphate dehydrogenase (G3PDH) were used as controls. Additional abbreviations: PRL, prolactin; GH, growth hormone; PL, placental lactogen; PLF-RP, proliferin-related protein; PLP, prolactin-like protein; d/tPRP, decidual/trophoblast prolactin-related protein.

ulatory roles of NK cells and the NK cell product IFNγ in the regulation of trophoblast invasion.

Materials and methods

Animals and tissue preparation

Holtzman rats were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA) and Tgε26, IFNγ null mutant, IFNγ receptor null mutant, and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Tgε26 mice are transgenic for the human CD3ε gene and are deficient in both T and NK cell lineages (Wang et al., 1994). A Tgε26 breeding colony was established by breeding hemizygous Tgε26 males with C57BL/6 females. Hemizygous Tgε26 mice were genotyped by PCR using primers to the human CD3ε gene (forward primer: 5′-CATATAAAGTCTCCATCTCTG-3′; reverse primer: 5′-TGCCCTCAGGTAGAGATAAAA-3′). Briefly, PCR was performed for 30 cycles with a denaturing temperature at 94°C (1 min), an annealing temperature at 50 °C (1 min), and an extension temperature of 72°C (1 min). Amplified products were resolved by agarose gel electrophoresis and stained with ethidium bromide. Both IFNγ and IFNγ receptor null mutant mouse colonies (Dalton et al., 1993; Huang et al., 1993) were each established on the C57BL/6 genetic background and maintained by homozygous $-/-$ male $\times$ homozygous $-/-$ female breeding.

To obtain timed pregnancies, females were caged overnight with fertile males. In the rat, the presence of sperm in the vaginal smear was designated as day 0 of pregnancy. In mice, day 1 of pregnancy was designated by the presence of a copulatory plug in the vagina. Male CD-1 mice were used to generate time pregnant hemizygous Tgε26 and wild type female mice. Pregnant IFNγ null mutant, IFNγ receptor null mutant, and C57BL/6 mice were each obtained by homozygous $\times$ homozygous breeding. Implantation sites, including uterus, metrial gland, and placental tissues, were dissected

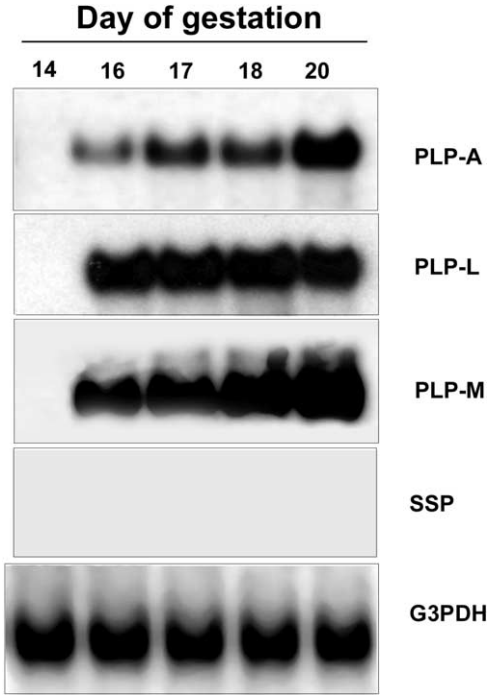


Fig. 3. Northern blot analyses of PLP-A, PLP-L, PLP-M, and SSP in the rat metrial gland. Total RNA was isolated from rat metrial gland on gestational days 14-20, fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon, and hybridized to [^{32}P]-labeled cDNA probes for PLP-A, PLP-L, PLP-M, SSP, or G3PDH. G3PDH was used to evaluate the integrity of the RNA samples and as a control for gel loading.

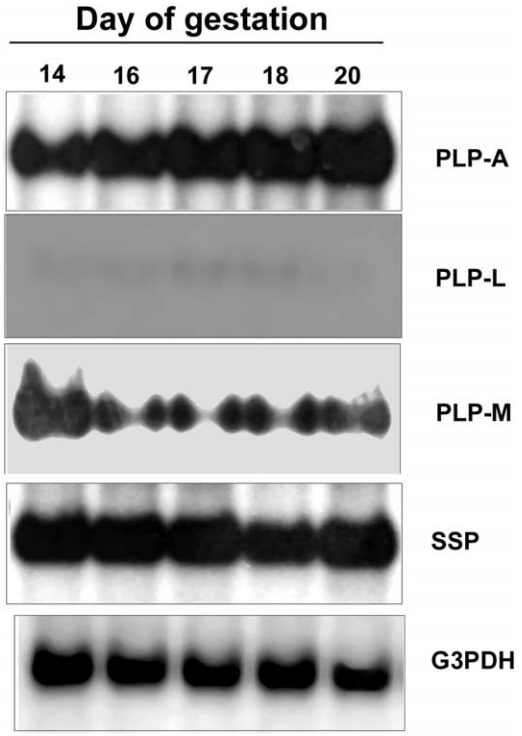


Fig. 4. Northern blot analyses of PLP-A, PLP-L, PLP-M, and SSP in the rat junctional zone of the chorioallantoic placenta. Total RNA was isolated from junctional zone tissues on gestational days 14-20, fractionated by formaldehyde-agarose gel electrophoresis, transferred to nylon, and hybridized to [^{32}P]-labeled cDNA probes for PLP-A, PLP-L, PLP-M, SSP, and G3PDH. G3PDH was used to evaluate the integrity of the RNA samples and as a control for gel loading.

from pregnant animals. Tissues were snap-frozen in liquid nitrogen for PRL family miniarray and Northern analysis. For in situ hybridization and immunocytochemistry, tissues were frozen in dry ice-cooled heptane. All tissue samples were stored at −80°C until used. The University of Kansas Medical Center Animal Care and Use Committee approved all procedures for handling and experimentation with rodents.

Immunocytochemistry

Immunocytochemical analyses were used for the purpose of identifying NK cells and trophoblast cells. All analyses were performed on 10-micron tissue sections prepared with the aid of a cryostat.

NK cells

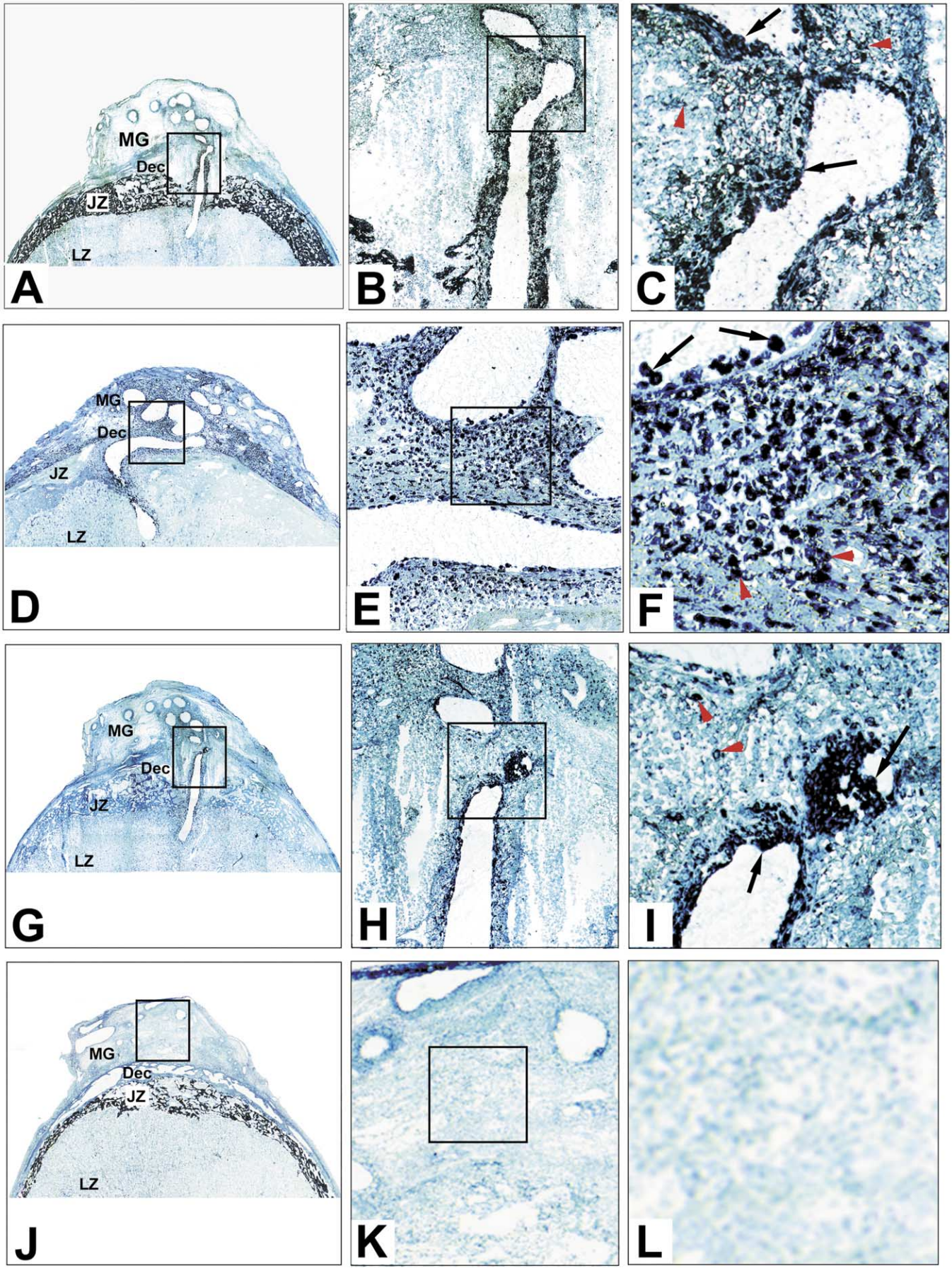
A rabbit polyclonal anti-rat perforin antibody (Torrey Pines Biolabs, Houston, TX) was used at a concentration of 2.5 μg/ml to detect NK cells in both rat and mouse tissues with a Histostain-SP kit (Zymed Laboratories, San Francisco, CA).

Trophoblast cells

Rat trophoblast cells were detected by using direct immunofluorescence staining with a fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-Pan cytokeratin antibody (Sigma Chemical Company, St. Louis, MO). Tissue sections were fixed in ice-cold acetone for 10 min and incubated with 10% normal rat serum in phosphate-buffered saline (PBS, 10 mM NaH_2PO_4, 150 mM NaCl, pH 7.4), for 15 min and then exposed for 30 min to FITC-conjugated antibody diluted in PBS. Slides were washed with several changes of PBS for 10 min. Stained tissue sections were examined and images recorded with a Nikon phase/epifluorescence microscope equipped with a CCD camera (Magnafire, Optronics, Goleta, CA). Mouse trophoblast cells were detected by using indirect immunofluorescence staining with a rat monoclonal anti-mouse cytokeratin antibody (TROMA-I; Developmental Studies Hybridoma Repository, Iowa City, IA). Tissue sections were fixed in ice-cold acetone for 10 min, incubated with 10% normal mouse serum in PBS for 15 min, and then exposed for 30 min to Troma-I. Samples were washed with several changes of PBS for 10 min, incubated for 30 min with Texas Red isothiocyanate (TRITC)-conjugated secondary mouse anti-rat IgG (Sigma Chemical Company, St. Louis, MO), and rinsed in PBS. Stained tissue sections were examined and images recorded as described above.

PRL family mini-array assay

The PRL family mini-array assay is a hybridization-based tool for simultaneously monitoring expression of each member of the PRL family (Dai et al., 2002). It has been effectively used to monitor the phenotypes of the placenta and trophoblast cells. The PRL family mini-array assay was performed as previously described (Dai et al., 2002). Twenty nanograms of PCR-amplified cDNA for each of the members of the PRL family was spotted, in duplicate, onto nylon membranes. Membranes were crosslinked and stored at 4°C until used. Total RNA was extracted from tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA). [α-P^{32}]dCTP-labeled cDNA probes were generated by reverse transcription using 5 μg of total RNA. Probes were purified by using micro bio-spin columns (Bio-Rad Laboratories, Richmond, CA). Membrane filters were briefly rinsed with water and prehybridized for 2 h at 42 °C with 5× SSPE (1 × SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, 10 mM EDTA, pH 7.4) containing 5 × Denhardt's reagent, 50% deionized formamide, 1% SDS, and salmon sperm DNA (100 μg/ml). Hybridizations were performed overnight with the labeled probes at 42 °C. Membranes were washed once with 2 × SSPE and 0.1% SDS for 30 min at 42°C and twice with 0.1× SSPE and 0.5% SDS at 60 C for 30 min each. Membranes were then wrapped with plastic wrap and exposed to Kodak Bio-Max film for 1–4 h and developed.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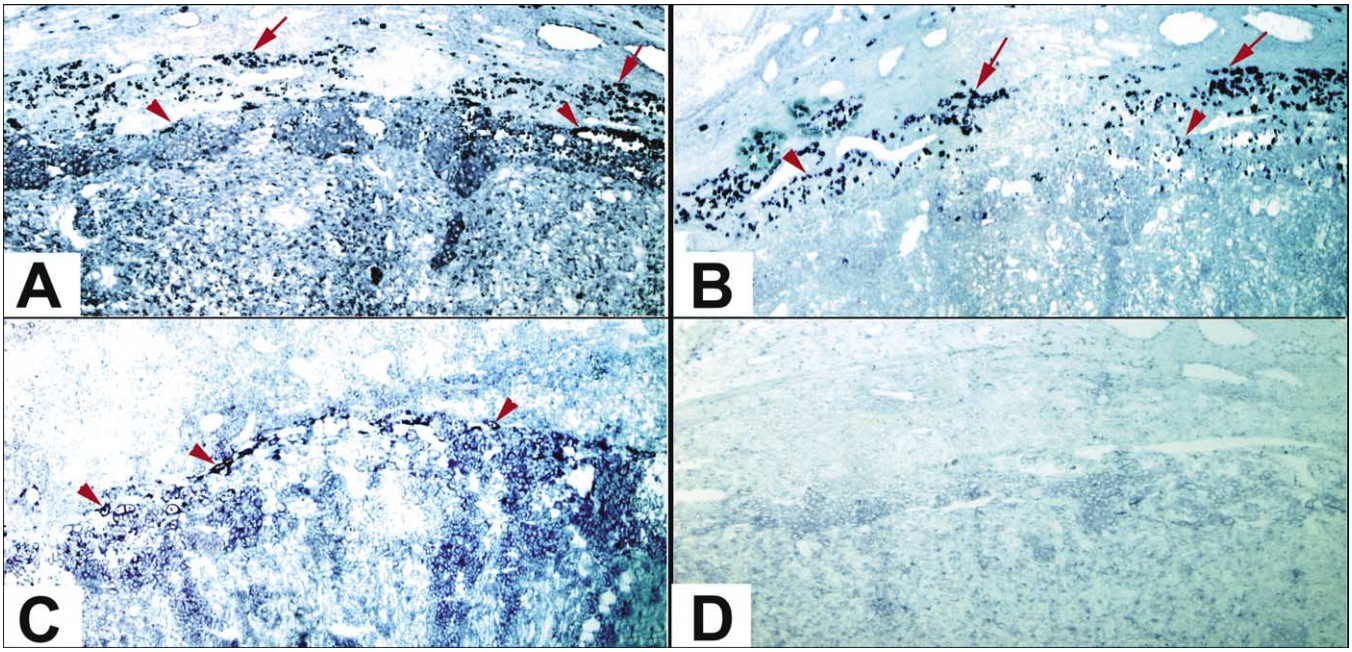


Fig. 6. Localization of PLP-M, PLP-N, and PLP-A mRNA at the maternal–fetal interface of the mouse. Mouse implantation sites were isolated at day 12 and day 18 of gestation, 10-micron cryosections of the tissue were prepared and hybridized to digoxigenin-labeled anti-sense and sense (data not shown) probes for PLP-M, PLP-N, or PLP-A. (A) PLP-M mRNA localization on day 18. (B) PLP-N mRNA localization on day 18. (C) PLP-A mRNA localization on day 12. (D) PLP-A mRNA localization on day 18. All magnifications are at 100 ×. Red arrows and red arrowheads indicate trophoblast giant cells and invasive trophoblast cells, respectively.

Northern blot analysis

Northern blot analysis was performed as described previously (Faria et al, 1990). Total RNA was extracted from tissues by using TRIzol reagent (Invitrogen). Total RNA (20 μg/lane) was resolved in 1% formaldehyde–agarose gels, transferred to nylon membranes, and crosslinked. Blots were probed with [α-P^{32}]-labeled cDNAs for PRL-like protein-A (PLP-A), PLPL, PLP-M (Dai et al., 2002), and spongiotrophoblast-specific protein (SSP; Iwatsuki et al., 2000). Glyceraldehyde-3′-phosphate dehydrogenase (G3PDH) cDNA was used to evaluate the integrity and equal loading of RNA samples. At least three different tissue samples from three different animals were analyzed with each probe for each time point.

In situ hybridization

In situ hybridization was performed as described previously (Braissant and Wahli, 1998; Wiemers et al. 2003). Ten-micron cryosections of tissues were prepared and stored at −80°C until used. Plasmids containing cDNAs for rat PLP-A, PLP-L, PLP-M, and SSP (Iwatsuki et al., 2000; Dai et al., 2002) and mouse PLP-A, PLP-L, PLP-M, and PLP-N (Müller et al., 1998; Wiemers et al., 2003) were used as templates to synthesize sense and anti-sense digoxigenin-labeled riboprobes according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Tissue sections were air dried and fixed in ice-cold 4% paraformaldehyde in PBS for 15 min. Prehybridization was carried out in a humidified chamber at 50°C in 5× SSC (standard saline citrate), 50% deionized formamide, 1× Denhardt's reagent, 10% dextran sulfate, and salmon sperm DNA (100 μg/ml). Hybridizations were performed in the same incubation conditions overnight. Slides were washed in 2× SSC at room temperature for 30 min followed by treatment with RNase-A (100 ng/ml) and additional washes with 2× SSC for 30 min at room temperature, with 2× SSC for 1 h at 65°C, with 0.1× SSC for 1 h at 65°C. Tissue samples were then blocked for 30 min and incubated with

Fig. 5. Localization of PLP-A, PLP-L, PLP-M, and SSP mRNAs at the maternal—fetal interface of the rat. Rat implantation sites were isolated at day 18 of gestation, 10-micron cryosections of the tissues were prepared and hybridized to digoxigenin-labeled anti-sense and sense (data not shown) probes for PLP-A (A–C), PLP-L (D–F), PLP-M (G–I); or SSP (J–L). PLP-A mRNA localization, 40 ×. (B) High magnification of (A), see boxed inset. (C) High magnification of (B), see boxed image. (D) PLP-L mRNA localization, 40 ×. (E) High magnification of (D), see boxed inset. (F) High magnification of (E), see boxed image. (G) PLP-M mRNA localization, 40 ×. (H) High magnification of (G), see boxed inset. (I) High magnification of (H), see boxed image. (J) SSP mRNA localization, 40 ×. (K) High magnification of (J), see boxed inset. (L) High magnification of (K) see boxed image. Black arrows and red arrowheads indicate endovascular and interstitial trophoblast cells, respectively. Abbreviations, MG, metrial gland; Dec, decidua; JZ, junctional zone; LZ, labyrinth zone.

alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500) in blocking buffer (Roche Molecular Biochemicals) for 2 h at room temperature. Slides were then washed and detection was performed by using nitro blue tetrazolium (250 μg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (225 μg/ml; Roche Molecular Biochemicals).

Assessment of trophoblast outgrowth

In vivo developed embryos were recovered from day 4 pregnant rats by flushing the excised uterine homs with 0.5 ml of equilibrated hamster embryo culture medium-2-modified (HECM-2m; Ain and Seshagiri, 1997) supplemented with MEM nonessential amino acids (Invitrogen). Only well formed fully expanded and hatched blastocysts were cultured in serum-coated dishes containing HECM-2m at 37°C with a humidified atmosphere of 5% CO_2 in air (Mishra and Seshagiri, 1998). A block design was used in which blastocysts from each animal were distributed randomly and equally into different treatments. Blastocysts were treated with rat interferon γ (IFNγ, PeproTech Inc, Rocky Hill, NJ), gestation day 13 rat material gland extract, rat IFNγ neutralizing antibodies (Research Diagnostics, Flanders, NJ), or isotype-matched control antibodies (Research Diagnostics Inc) for 72 h. Antibodies were used at a final concentration of 5 μg/ml. Metrial gland extracts were prepared by homogenizing metrial glands in sterile prechilled Hank's balanced salt solution (10 metrial glands/ml). The supernatant was recovered by centrifugation, 15 min at 16,000 *g*, and stored at −70°C until used. Metrial gland extracts were used at a final dilution of 1:10 (vol:vol). A minimum of eight replicate experiments was performed. Attachment was verified by the inability to dislodge blastocysts following agitation. Trophoblast outgrowth was monitored and images were recorded with a Nikon phase contrast microscope attached with a CCD camera (Magnafire Optronics). The area of outgrowth was measured by using image analysis software, Scion Image (Scion Corp., Frederick, MA).

Statistical analysis

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

Results

Identification of invasive trophoblast cells in the uterus of the rat and mouse

Previous work in the rat and mouse described the presence of extraplacental trophoblast cells (Bridgman, 1949; Redline and Lu, 1989). These early reports relied primarily on using morphological features of the trophoblast cells for their identification. We took advantage of the epithelial nature of trophoblast cells and used antibodies to cytokeratins for the purpose of identifying and localizing trophoblast cells (Hunt and Soares, 1988; Kruse et al., 1999) within the mesometrial compartment of the rat and mouse uterus. On day 18 of gestation in the rat, cytokeratin-positive cells were present in the chorioallantoic placenta and were dispersed throughout the mesometrial decidua and metrial gland (Fig. 1A and B). Both interstitial and endovascular trophoblast cells were evident in the rat metrial gland. In the mouse, extraplacental cytokeratin-positive cells were primarily restricted to the mesometrial decidua (Fig. 1C and D). The preferred mode of invasion into the decidua of the mouse was interstitial with a lesser endovascular contribution.

Differentiated endocrine phenotype of invasive trophoblast cells in the rat

Since cytokeratin-positive/trophoblast cells were identified within the metrial gland of the rat and since the metrial gland can be easily dissected, we next used dissected metrial gland tissues to assess the endocrine phenotype of the invasive trophoblast cells. The PRL family miniarray assay was performed on RNA samples isolated from metrial gland and placental tissues dissected on day 18 of gestation (Fig. 2). The metrial gland expressed a subset of three PRL family members, PLP-A, PLP-L, and PLP-M (Fig. 2). In contrast, the junctional zone of the placenta expresses mRNAs for 12 different family members (Fig. 2). Two of the 3 PRL family members expressed in the metrial gland, PLP-A and PLP-M, were also expressed in the placenta. PLP-L expression was restricted to the metrial gland. Gestational profiles of PLP-A, PLP-L, and PLP-M mRNA expression were determined by Northern analysis. Expression of PRL family members in the metrial gland was first detected on day 16 of gestation (Fig. 3). PLP-A and PLP-M mRNA levels showed a gradual increase as gestation advanced, whereas PLP-L mRNA levels were consistently elevated from day 16 to day 20 of gestation. PLP-A and PLP-M mRNAs were expressed in junctional zone placental tissues from day 14 to day 20 of gestation. PLP-L mRNA expression in the junctional zone was below the limit of detection throughout the time period of analysis (Fig. 4). Additionally, the metrial gland did not express SSP mRNA, a spongiotrophoblast cell-specific transcript, abundantly expressed in the junctional zone of the placenta.

Transcripts for PLP-A, PLP-L, PLP-M, and SSP were localized by in situ hybridization (Fig. 5). PLP-A and PLP-M were localized to trophoblast cells in the junctional zone and to both endovascular and interstitial trophoblast cells of the mesometrial compartment (Fig. 5A–C, G–I). Placental expression of PLP-A was limited to trophoblast giant cells and spongiotrophoblast cells in the junctional zone, whereas PLP-M mRNA was observed in these same

junctional zone trophoblast components, and a lower level of expression in some labyrinthine trophoblast cells (data not shown). Trophoblast cell plugs expressing PLP-M were observed within blood vessels (Fig. 5I). PLP-L mRNA localization was restricted to endovascular and interstitial cells of the mesometrial compartment (Fig. 5D–F), whereas SSP mRNA was restricted to the spongiotrophoblast of the junctional zone (Fig. 5J–L). The specificity of the hybridizations was determined by using sense riboprobes, which failed to detect reproducible hybridization signals in any of the tissue sections (data not shown).

Trophoblast cell invasion in the mouse: PRL family gene expression

As noted above, trophoblast cell invasion into the mesometrial compartment is not as deep in the mouse as it is in the rat. Trophoblast invasion was confined primarily to the tightly adherent mesometrial decidua overlying the chorioallantoic placenta. Thus, we were not able to isolate tissues containing invasive trophoblast for biochemical analyses in the mouse. We did, however, examine the expression pattern of PRL family members by in situ hybridization. PLP-M and PLP-N mRNA-positive cells were identified within the junctional zone of the chorioallantoic placenta in both trophoblast giant cells and spongiotrophoblast cells and extended to locations throughout the mesometrial uterine decidua (Fig. 6A and B). Unlike the rat, PLP-A (Fig. 6C and D) and PLP-L (Wiemers et al., 2003; data not shown) mRNAs were not detected in invasive mouse trophoblast cells.

Reciprocal relationship between NK cells and invasive trophoblast cells

In addition to trophoblast cells, the mesometrial compartment has another prominent cellular resident, NK cells (Croy et al., 1996; Head, 1996). NK cells are distributed within interstitial and perivascular regions of the mesometrial compartment. We therefore explored the possible relationship between NK cells and invading trophoblast cells. Cytokeratin immunostaining was used to track trophoblast cells, and NK cells were monitored by immunocytochemistry for perforin, a protein located within granules of NK cells. Trophoblast cell invasion into the uterine decidua was first initiated between days 14 and 15 of gestation in the rat and on day 14 of gestation in the mouse and progressively increased as gestation proceeded (data not shown). Trafficking of NK cells and trophoblast cells within the mesometrial compartment were inversely related in both the rat (Fig. 7A–D) and the mouse (Fig. 7E–H). Trophoblast cell invasion occurred following the demise of NK cells and was directed toward regions of the mesometrial compartment previously occupied by NK cells. After day 18, the only remaining uterine NK cells were restricted to the lateral aspects of the metrial gland.

NK cells influence trophoblast invasion

Given the reciprocal relationship between NK cells and invading trophoblast cells within the mesometrial compartment of both the rat and mouse, we explored the role of NK cells as potential regulators of trophoblast cell invasion. NK cells represent the primary type of lymphocyte present in the uterus (Croy et al., 1998). Trophoblast cell invasion was investigated in wild type and in Tgε26 (transgenic mice deficient in NK cells) mice (Fig. 8). The mesometrial compartment of pregnant Tgε26 mutant mice possess very few NK cells in their mesometrial decidua and metrial gland on day 13 of gestation (Fig. 8C), in contrast to the large number of NK cells in these compartments of wild type mouse (Fig. 8A). The NK cell deficiency in Tgε26 mice was correlated with an accelerated migration of cytokeratin-positive and PLP-M-positive cells into the mesometrial decidua (Fig. 8D and F). This observation contrasted with minimal trophoblast cell invasion in wild type mice on day 13 of gestation (Fig. 8B and E). These experiments provide genetic evidence for a linkage between NK cells and trophoblast cell invasion.

IFNγ signaling and trophoblast invasion

IFNγ is a prominent secretory product of activated NK cells, including those residing in the mesometrial compartment of the uterus (Ain et al., 2003). Furthermore, IFNγ has been proposed to be the principle mediator of NK cell function within the uterus during pregnancy (Ashkar and Croy, 2001). Consequently, we examined the effect of IFNγ on the outgrowth of trophoblast cells from cultured blastocysts. IFNγ did not affect substrate attachment of zona-free blastocysts; however, IFNγ significantly inhibited the outgrowth of blastocysts and caused degenerative morphological changes (Table 1;Fig. 9A–D). The effective IFNγ concentration required for inhibition of trophoblast outgrowth was high relative to the concentration of IFNγ in the uterine mesometrial compartment (Ashkar and Croy, 1999; Ain et al., 2003). Consequently, additional experiments were performed to further evaluate a role for IFNγ signaling in the regulation of trophoblast invasion. The effects of extracts prepared from gestation day 13 metrial glands on trophoblast outgrowth were evaluated (Table 2;Fig. 9A, E, and F A, E, and F). Metrial gland extracts inhibited trophoblast outgrowth. The inhibition was partially reversed by coincubation with neutralizing antibodies to rat IFNγ (Table 2; Fig. 9E and F). Finally, the onset of trophoblast invasion was investigated in IFNγ null mutant and IFNγ receptor null mutant mouse lines. Evidence for an early onset of trophoblast invasion was observed in both mutant mouse lines (Fig. 10). The results indicate that IFNγ may participate in the NK cell modulation of trophoblast cell invasion.

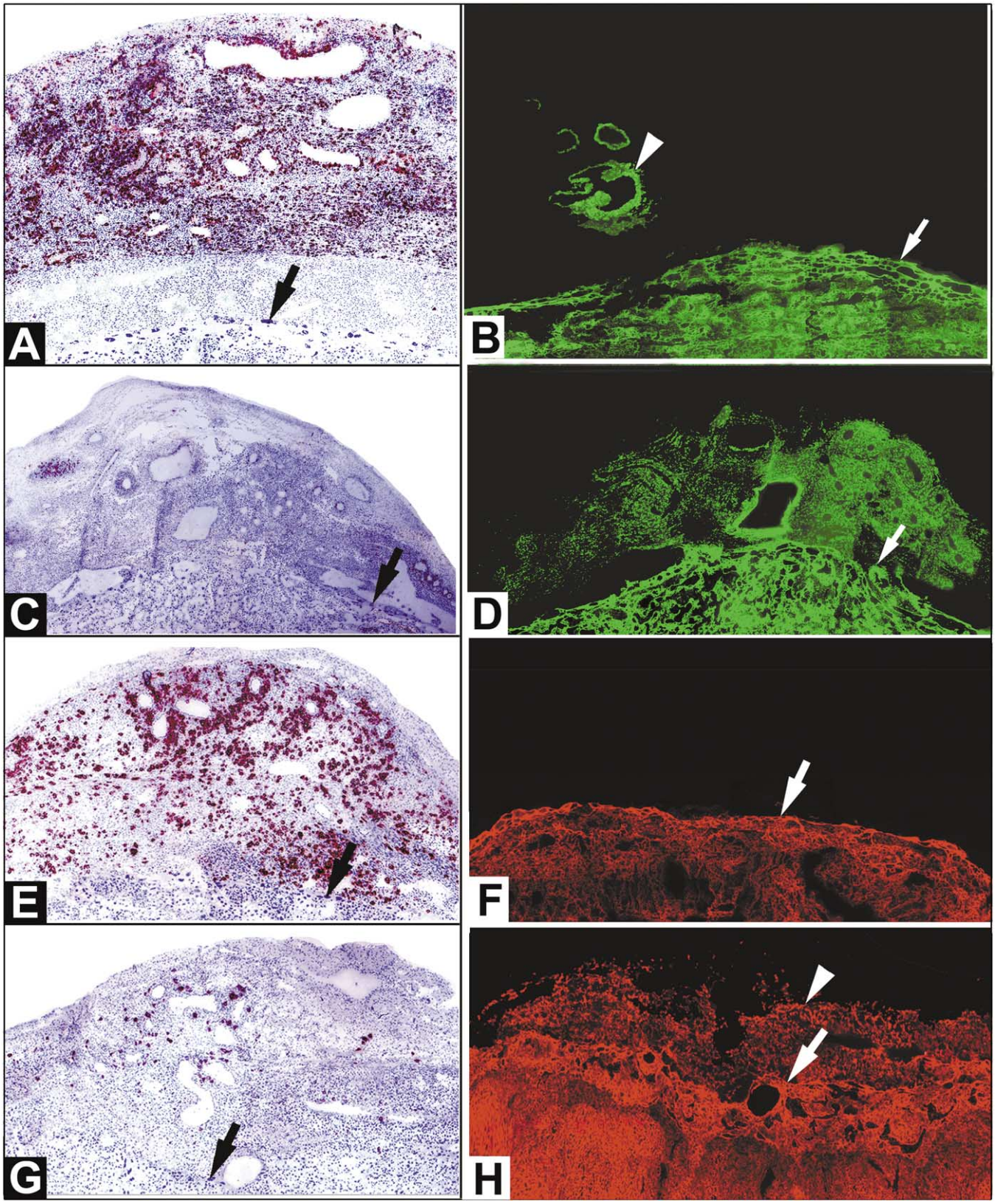


Fig. 7. Inverse spatial and temporal relationship between NK cells and invasive trophoblast cells at the maternal–fetal interface of the rat and mouse. Immunocytochemical localization of NK cells (perforin) and trophoblast cells (cytokeratin) on gestational days 14 and 18 for the rat (A–D) or gestational days 13 and 18 for the mouse (E–H). (A) Localization of NK cells on day 14 of gestation. (B) Localization of trophoblast cells on day 14 of gestation. (C) Localization of NK cells on day 18 of gestation. (D) Localization of trophoblast cells on day 18 of gestation. (E) Localization of NK cells on day 13 of gestation. (F) Localization of trophoblast cells on day 13 of gestation. (G) Localization of NK cells on day 18 of gestation. (H) Localization of trophoblast cells on day 18 of gestation. Magnifications for (A–D) are 100 × and (E–H) are at 200 ×. The arrows indicate the trophoblast giant cell boundaries between the chorioallantoic placenta and decidua, and the arrowhead indicates the location of the invasive trophoblast cells.

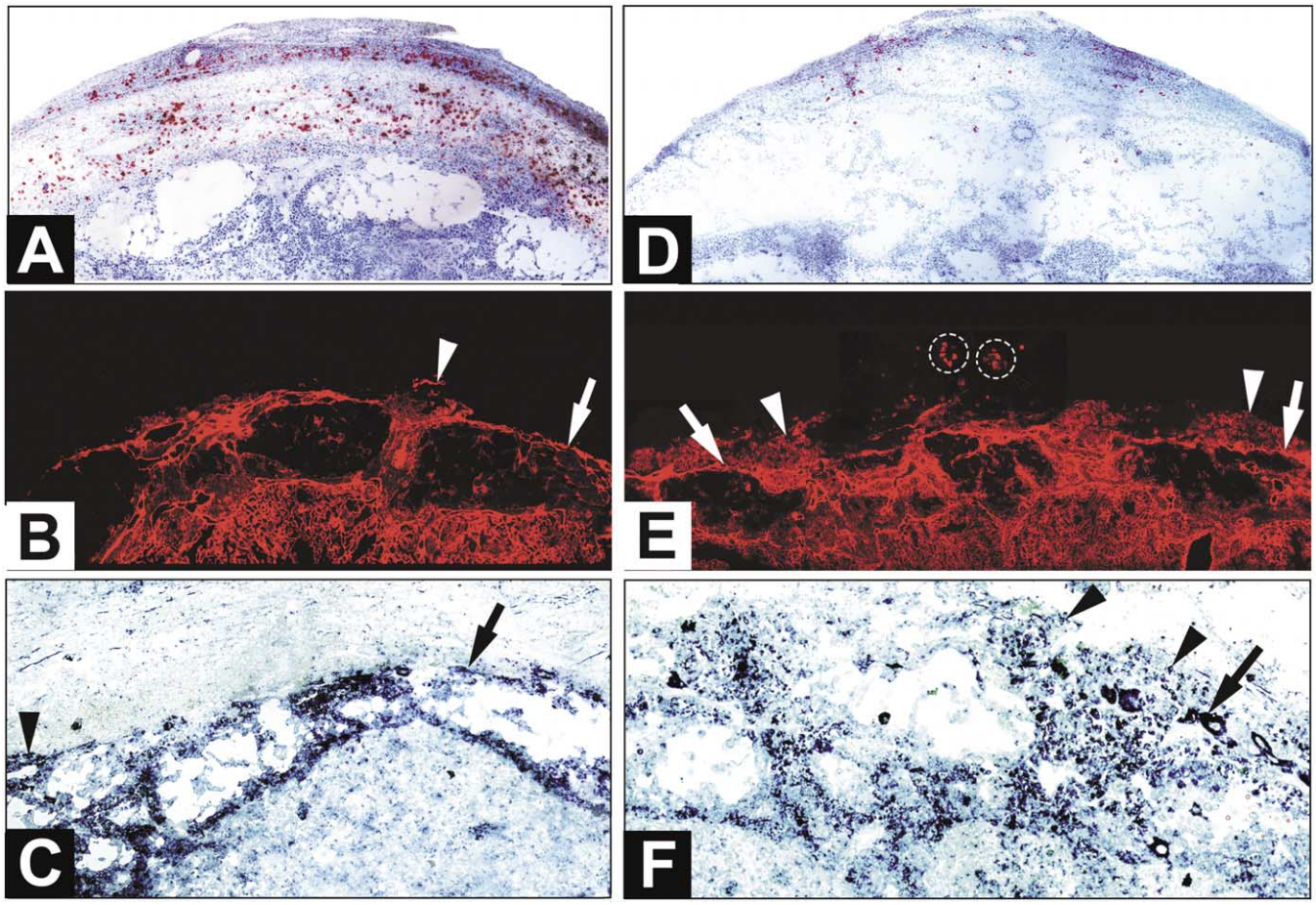


Fig. 8. Acceleration of trophoblast invasion in NK cell deficient mice. Localization of NK cells (immunocytochemistry for perforin) and trophoblast cells (immunofluorescence for TROMA-I, cytokeratin, or in situ hybridization for PLP-M mRNA) in implantation sites from gestational day 13 of normal and NK cell deficient Tgε26 mouse. (A) Localization of NK cells in wild type mice. (B) Localization of trophoblast cells (cytokeratin) in wild type mice. (C) Localization of PLP-M mRNA in wild type mice. (D) Localization of NK cells in NK cell deficient mice. (E) Localization of trophoblast cells (cytokeratin) in NK cell–deficient mice. Please note that the encircled structures represent migratory trophoblast cells penetrating deep into the uterine decidua. (F) Localization of PLP-M mRNA in NK cell–deficient mice. Magnifications for panels (A) and (D) are at 40 × and (B), (C), (E), and (F) are at 100 ×. The arrows indicate the trophoblast giant cell boundaries between the chorioallantoic placenta and decidua, and the arrowheads indicate the location of additional invasive trophoblast cells near the decidua–placenta interface.

Discussion

Trophoblast cells facilitate embryonic/fetal development. They accomplish this task in a variety of ways. First and foremost, trophoblast cells differentiate into multiple specialized cell types. This multilineage pathway is particularly evident in rodents. Trophoblast cells arise that are specialized for nutrient transfer, energy storage, hormone produc-

Table 1
Influence of IFNγ on rat trophoblast outgrowth

Treatment	No. of blastocysts cultured	No. of blastocysts attached	No. of blastocysts with outgrowth	Surface area of outgrowth[a] ($\times 10^{-3}$ μm^2)
Control	31	31	31	17.8 ± 2.9[b]
IFNγ (10^3 U/ml)	30	30	30	16.9 ± 5.2
IFNγ (5×10^3 U/ml)	31	31	31	11.3 ± 5.7
IFNγ (10^4 U/ml)	31	31	19	1.9 ± 1.7[c]

[a] In the IFNγ treatment group, blastocysts that did not show outgrowth were not included for surface area measurement.
[b] Values represent means ± standard error of the mean.
[c] Control versus IFNγ treatment, $P < 0.01$.

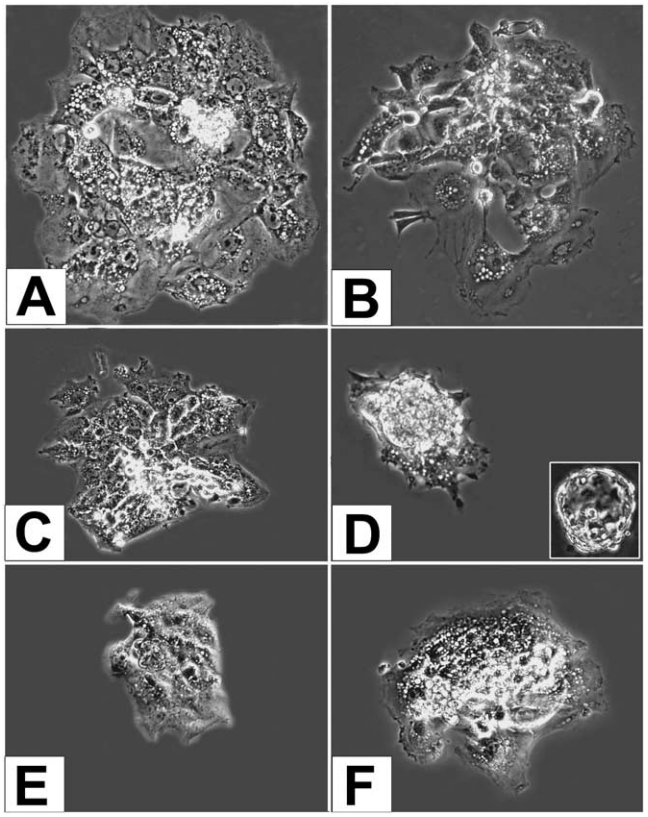


Fig. 9. The effect of IFNγ on blastocyst trophoblast outgrowth. In vivo developed zona-free rat blastocysts were cultured with various treatments for 72 h. Surface areas of the outgrowths were measured and are presented in Tables 1 and 2. Representative images of trophoblast cell outgrowths from blastocyst cultures in control medium (A), and following treatment with 10^3 units/ml IFNγ (B), 5×10^3 units/ml IFNγ (C), 10^4 units/ml IFNγ (D), day 13 metrial gland extract (E), or day 13 metrial gland extract + IFNγ neutralizing antibody (5 μg/ml; F). Please note that some IFNγ-treated blastocysts showed limited outgrowth, whereas others failed to show outgrowth (D, inset). All magnifications are at 100×.

tion, and invasion. In this report, we show that a subpopulation of rodent trophoblast cells escape from the chorioallantoic placenta, enter the uterine decidua, and in the rat, extend into the myometrium. These cells penetrate and surround uterine blood vessels and possess the capacity for hormone production. The timing of the placental exit is precise and coincides with the disappearance of uterine mesometrial NK cells.

Trophoblast cell invasion is both endovascular and interstitial (Pijnenborg et al., 1981; Georgiades et al., 2002). In the rat, intraplacental endovascular trophoblast cells can be identified at midgestation and expand into blood vessels situated in the uterine decidua and subsequently into the myometrium as gestation advances (present study). Invading interstitial trophoblast cells similarly penetrate the uterine decidua and invade into the myometrium; however, their movements are restricted to the last third of gestation. Trophoblast cell invasion is more limited in the mouse and confined to the uterine mesometrial decidua. In the human, interstitial trophoblast cell invasion begins postimplantation and is completed by 7–9 weeks of gestation and is limited to the decidua; endovascular invasion is initiated around week 9 and completed by week 22 (Georgiades et al., 2002). The rat and mouse model systems exhibit features shared with primates and expand our experimental repertoire for studying trophoblast cell invasion.

The phenotype of invasive rodent trophoblast cells is distinct from phenotypes for intraplacental trophoblast cell lineages. In early reports, invasive trophoblast cells were characterized by their morphology with trophoblast giant cells and glycogen cells being the primary trophoblast lineages recognized (Bridgman, 1949; Redline and Lu, 1989; Teesalu et al., 1998; Zybina et al., 2000; Zybina and Zybina, 2000). Invasive trophoblast cells do not express the complement of gene markers diagnostic of trophoblast giant cells, spongiotrophoblast cells, glycogen cells, or syncytial trophoblast cells residing in the chorioallantoic placenta. In the present report, we determined that invasive trophoblast cells possess a hormone/cytokine producing activity. Rat endovascular and interstitial trophoblast cells migrating into the metrial gland express a subset of members of the PRL gene family, consisting of PLP-A, PLP-L, and PLP-M. PLP-A and PLP-M are dually expressed in the invasive trophoblast cells and by trophoblast lineages in the chorioallantoic placenta, whereas during the latter third of pregnancy, PLP-L expression is restricted to invasive trophoblast cells of the metrial gland. The rat ortholog of PLP-N has recently been identified and its expression is restricted to invasive trophoblast cells, as well (S. Ohboshi, R.A., D.O. Wiemers, and M.J.S., unpublished observations). Although mouse invasive trophoblast cells express PLP-M and PLP-N, unlike the rat, they do not express PLP-A or PLP-L (present study; Wiemers et al., 2003). Expression of PLP-A and PLP-L is restricted to the junctional zone of the mouse

Table 2
Influence of gestation day 13 metrial gland extracts on rat trophoblast outgrowth

Treatment	No. of blastocysts cultured	Surface area of outgrowth ($\times 10^{-3}$ μm^2)
Control	28	16.9 ± 3.1[a]
Metrial gland extract (MG-Ext)	32	3.9 ± 1.7[b]
MG-Ext + IFNγ neutralizing antibody	32	7.6 ± 1.1[c]
Control + isotype-matched control antibody	27	17.1 ± 1.8
MG-Ext + isotype-matched control antibody	28	4.1 ± 1.3[b]

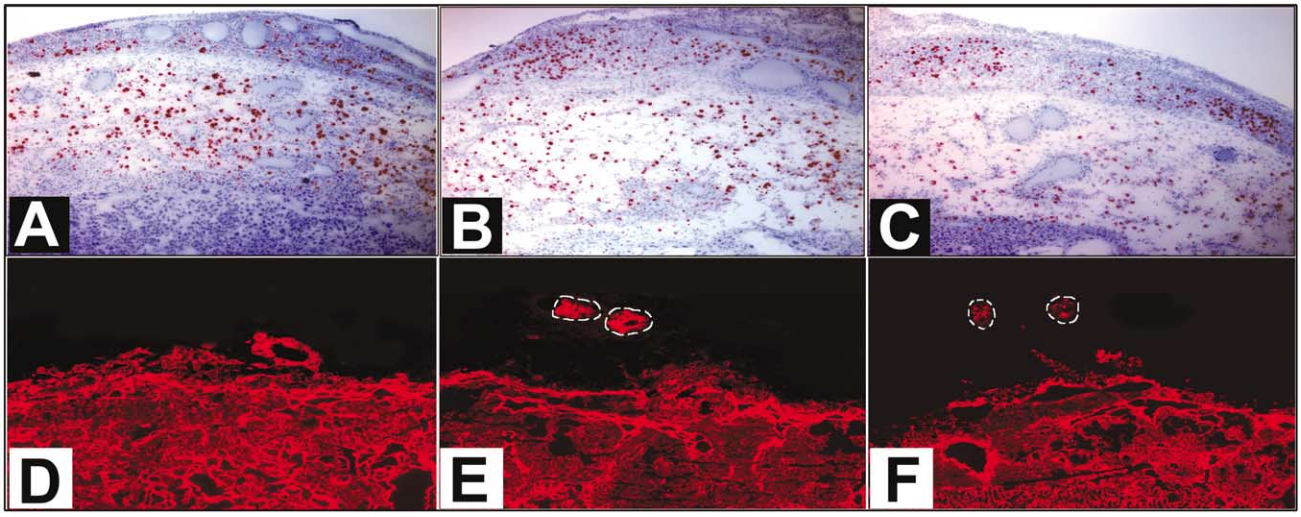
[a] Values represent means ± standard error of the mean.
[b] Control versus metrial gland extract, $P < 0.01$.
[c] Metrial gland extract + IFNγ neutralizing antibody versus metrial gland extract with or without isotype-matched control antibody, $P < 0.05$.

chorioallantoic placenta (Wiemers et al., 2003). PLP-L and PLP-M are also expressed in intraplacental endovascular trophoblast cells of the rat (Toft and Linzer, 2000; present study). Invasive interstitial trophoblast cells express other marker genes, including insulin like growth factor-II (Redline et al., 1993; Correia-da-Silva et al., 1999), p57KIP2 (Geogiades et al., 2001), urokinase type plasminogen activator (Teesalu et al., 1998), and class I major histocompatibility complex antigens (Redline and Lu, 1989).

Endocrine functions of the metrial gland were proposed many years ago. Selye and McKeown (1935) designated the mesometrial compartment of the rat as the "metrial gland" and discussed its possible endocrine activities. Others have discounted these early speculations and focused on the immune cell populations and vasculature of the mesometrial compartment (Croy, 1999; Stewart, 1999, 2001; Pijnenborg, 2000). The metrial gland is a dynamic structure and it is now apparent that in the rat resident invasive trophoblast cells produce members of the PRL family of hormones/cytokines (present study). In contrast to the rat, the mouse metrial gland does not possess the same endocrine activities. Invasive trophoblast cells of the mouse uterine decidua produce members of the PRL family (Wiemers et al., 2003, present study); however, these cells do not penetrate into the metrial gland. Interstitial and endovascular trophoblast cells of the rat metrial gland are well positioned for the secretion of factors, which can affect maternal physiology. Although, the biological actions of PLP-L, PLP-M, and PLP-N have not yet been reported, some insights into the biology of PLP-A have been described. PLP-A is present in maternal circulation (Deb et al., 1989, 1990), specifically targets uterine NK cells, and modulates NK cell function, including inhibition of cytotoxicity and cytokine production (Müller et al., 1999; Ain et al., 2003). Whether PLP-A also coordinates NK cell trafficking within the mesometrial compartment remains to be determined. Resident immune cells, the vasculature and the myometrium of the mesometrial compartment are additional putative targets for the paracrine actions of PLP-A, PLP-L, PLP-M, and PLP-N.

The exit of trophoblast cells from the chorioallantoic placenta and their movements into the uterine stroma and/or myometrium is temporally and spatially well defined. The precision of these events suggests that they are regulated. In this report, we demonstrate a reciprocal relationship in the trafficking of NK cells and trophoblast cells into the mesometrial compartment (Fig. 11). NK cells are the primary lymphocyte population within the uterus during pregnancy (Croy et al., 1998). They expand in the mesometrial compartment during the immediate postimplantation period where they associate and contribute significantly to pregnancy-associated mesometrial decidual and vascular remodeling (Peel, 1989; Head, 1996; Guimond et al., 1997; Croy et al., 1998, 2000; Wang et al., 2003). During the last week of pregnancy (beginning around day 14–15 of gestation in the mouse and rat), mesometrially located uterine NK cells begin to disappear, at least in part, via the initiation of apoptosis (Peel, 1989; Fukazawa et al., 1998). As NK cells disappear from the mesometrial compartment, trophoblast cells begin invading. Furthermore, interstitially invading trophoblast cells position themselves in locations previously occupied by NK cells. These observations indicate a potentially intriguing regulatory role for NK cells in trophoblast cell invasion and/or alternatively a role for invasive trophoblast cells in the demise of uterine NK cells. NK cell-deficient mouse strains have been generated and offer an opportunity for evaluation of NK cell-trophoblast cell trafficking dynamics. NK cell deficiency is associated with defects in mesometrial blood vessel remodeling (Guimond et al., 1997; Croy et al., 2000) and an alteration in the timing of trophoblast cell invasion (present study). Invasive trophoblast cells entered the uterine decidua at an earlier time point in NK cell deficient mice than they did in mice with normal complements of NK cells. Once trophoblast cells invaded into the mesometrial decidua, their spatial locations were similar in NK cell deficient and normal mice. NK cells and invasive interstitial trophoblast cells may possess overlapping actions in the mesometrial compartment. The early onset of interstitial trophoblast cell invasion in NK cell deficient mice suggests that invasive trophoblast cells may functionally compensate for an NK cell deficiency. The reciprocal relationship between NK cell and trophoblast cell trafficking in the mesometrial compartment also suggests that NK cells may produce factors that modulate trophoblast cell invasion.

The actions of NK cells on trophoblast cell invasion are likely mediated by NK cell secretory products, including cytokines, and may be direct or indirect. Uterine NK cells produce several cytokines and are the primary source of IFNγ in the metrial gland (Ashkar and Croy, 1999; Ain et al., 2003). IFNγ has been implicated as a major mediator of uterine NK cell function during pregnancy (Ashkar et al., 2000; Ashkar and Croy, 2001). Trophoblast cells are among a variety of different IFNγ targets. In vitro trophoblast cell differentiation, survival, and outgrowth are affected by IFNγ (Haimovici et al., 1991; Athanassakis et al., 2000; Pijnenborg et al., 2000; present study). Consistent with a role of NK cell IFNγ in the modulation of trophoblast cell invasion, we found that metrial gland extracts and IFNγ inhibited in vitro trophoblast outgrowth and that immunoneutralization with IFNγ antibodies partially reversed the inhibitory effects of the metrial gland extracts (present study). Furthermore trophoblast cell invasion exhibited an earlier onset in IFNγ and IFNγ receptor null mutants. The inhibitory actions of IFNγ may be accentuated by the synergistic activity of additional cytokines originating from uterine NK cells and/or other cells of the uterine mesometrial compartment (Pijnenborg et al., 2000). Alternatively, NK cells and/or IFNγ may also influence trophoblast cell invasion indirectly through their effects in remodeling the mesometrial decidual extracellular matrix and vascular compartment (Ashkar and Croy, 2001). The results generated in this report are consistent with a role for NK cells in



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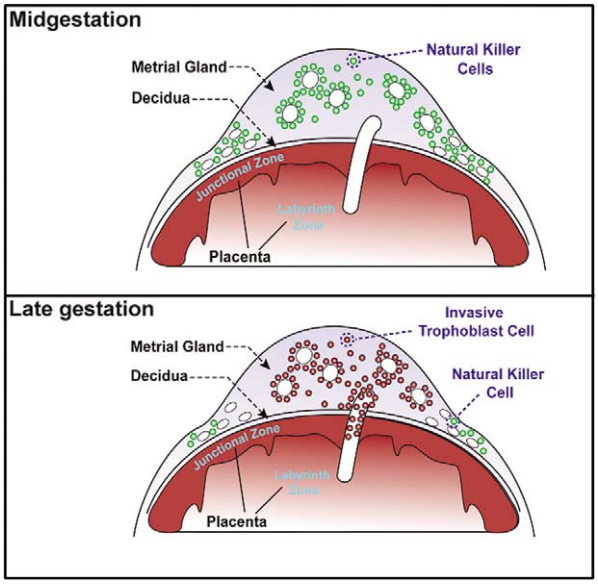

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Fig. 10. Trophoblast invasion in IFNγ and IFNγ receptor null mutant mice. Localization of NK cells (immunocytochemistry for perforin; A–C) and trophoblast cells (immunofluorescence for cytokeratin; D–F) in implantation sites from gestational day 13 wild type (A and D), IFNγ null mutant (B and E), and IFNγ receptor null mutant mice (C and F). Magnification for (A–C) are at 40 × and magnifications for (D–F) are at 100 ×. Please note that the encircled structures represent migratory trophoblast cells penetrating deep into the uterine decidua. The arrows indicate the trophoblast giant cell boundaries between the chorioallantoic placenta and decidua, and the arrowheads indicate the location of additional invasive trophoblast cells near the decidua-placenta interface.
Fig. 11. Schematic representation showing gestational-dependent changes of trophoblast cell invasion in the rat. The mesometrial compartment of the uterus is depicted at midgestation and during the latter stages of pregnancy. Please note the reciprocal relationship between the presence of NK cells and invasive trophoblast cells. Trophoblast cells migrate into regions previously occupied by NK cells. Both endovascular and interstitial invasive trophoblast cells are shown.

the modulation of interstitial trophoblast cell invasion and a role for IFNγ as a contributor to the dialog between uterine NK cells and trophoblast cells.

It is apparent that NK cells and IFNγ are not the only regulators affecting the entry of trophoblast cells into the uterine mesometrial compartment. A “seed” and “soil” analogy may be appropriate. Trophoblast cells likely need to acquire a state of competence for their exit from the chorioallantoic placenta and the uterine mesometrial compartment likely requires changes necessary for it to become receptive to the invading trophoblast cells. The demise of NK cells may contribute to the acquisition of uterine mesometrial receptivity. Interference with NK and IFN signaling affected the timing of trophoblast cell invasion but not the depth of trophoblast cell invasion into the uterus. The results imply that there are additional regulatory factors (cytokines, growth factors, extracellular matrix composition, etc.) participating in the control of trophoblast cell invasion. Colony stimulating factor-I, epidermal growth factor, hepatocyte growth factor, and insulin-like growth factor-I have been implicated as stimulators of primate trophoblast cell invasion (Bass et al., 1994; Hamilton et al.,

1998; Nasu et al., 2000; Aplin et al., 2000). Whether these factors also participate in the regulation of trophoblast cell invasion in the rat or mouse remains to be determined.

In conclusion, rodent trophoblast cells exhibit invasive behavior during the latter stages of pregnancy. They exhibit a unique endocrine phenotype and factors present in the uterine mesometrial compartment modulate their migratory behavior. Trophoblast cell invasion has elements of species-specificity and is more extensive in the rat than in the mouse. Most importantly, trophoblast cell invasion in both rodent species has parallels with invasive processes occurring during placentation in other species, including primates.

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