Interactions between Trophoblast Cells and the Maternal and Fetal Circulation in the Mouse Placenta

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Mammalian embryos have an intimate relationship with their mothers, particularly with the placental vasculature from which embryos obtain nutrients essential for growth. It is an interesting vascular bed because maternal vessel number and diameter change dramatically during gestation and, in rodents and primates, the terminal blood space becomes lined by placental trophoblast cells rather than endothelial cells. Molecular genetic studies in mice aimed at identifying potential regulators of these processes have been hampered by lack of understanding of the anatomy of the vascular spaces in the placenta and the general nature of maternal-fetal vascular interactions. To address this problem, we examined the anatomy of the mouse placenta by preparing plastic vascular casts and serial histological sections of implantation sites from embryonic day (E) 10.5 to term. We found that each radial artery carrying maternal blood into the uterus branched into 5–10 dilated spiral arteries located within the metrial triangle, populated by uterine natural killer (uNK) cells, and the decidua basalis. The endothelial-lined spiral arteries converged together at the trophoblast giant cell layer and emptied into a few straight, trophoblast-lined “canals” that carried maternal blood to the base of the placenta. Maternal blood then percolated back through the intervillous space of the labyrinth toward the maternal side of the placenta in a direction that is countercurrent to the direction of the fetal capillary blood flow. Trophoblast cells were found invading the uterus in two patterns. Large cells that expressed the trophoblast giant cell-specific gene Plf (encoding Proliferin) invaded during the early postimplantation period in a pattern tightly associated with spiral arteries. These peri/endovascular trophoblast were detected only 150–300 μm upstream of the main giant cell layer. A second type of widespread interstitial invasion in the decidua basalis by glycogen trophoblast cells was detected after E12.5. These cells did not express Plf, but rather expressed the spongiotrophoblast-specific gene Tpbp. Dilation of the spiral arteries was obvious between E10.5 and E14.5 and was associated with a lack of elastic lamina and smooth muscle cells. These features were apparent even in the metrial triangle, a site far away from the invading trophoblast cells. By contrast, the transition from endothelium-lined artery to trophoblast-lined (hemochorial) blood space was associated with trophoblast giant cells. Moreover, the shaping of the maternal blood spaces within the labyrinth was dependent on chorioallantoic morphogenesis and therefore disrupted in Gcm1 mutants. These studies provide important insights into how the fetoplacental unit interacts with the maternal intraterine vascular system during pregnancy in mice. © 2002 Elsevier Science (USA)

Key Words: labyrinth; mouse; placenta; pregnancy; spiral artery; trophoblast; umbilical artery; uterus; vascularization; vascular corrosion casts.

INTRODUCTION

The maternal vascular bed of the uterus changes dramatically during pregnancy as existing vessels dilate and new vessels form (Cross et al., 2002; Pijnenborg et al., 1981). In addition, in primates and rodents, the terminal vascular bed is not lined by endothelial cells but is rather hemochorial, meaning that maternal blood perfuses a space lined by trophoblast cells (Wooding and Flint, 1994). Appropriate perfusion of this space is necessary for critical endocrine and exchange functions both to sustain normal growth and development and to provide for the needs of the developing embryo. To understand the function of the placenta, it is essential to understand the nature of the maternal-fetal interface and the exchanges that occur there. The placenta is the site of all fetal-maternal exchange and is the organ that connects the two individuals in a specific and intimate way during pregnancy. The placenta must provide a unique environment for development, including the provision of nutrients, oxygen, and waste removal in reverse direction to those of normal maternal circulation. A better understanding of the development of the placenta may increase knowledge of the normal range of placental functioning and may provide insights into pathologies that arise from placental defects. Understanding the function of the placenta requires a knowledge of the anatomy of the placental vasculature. Molecular genetic studies in mice aimed at identifying potential regulators of these processes have been hampered by lack of understanding of the anatomy of the vascular spaces in the placenta and the general nature of maternal-fetal vascular interactions. To address this problem, we examined the anatomy of the mouse placenta by preparing plastic vascular casts and serial histological sections of implantation sites from embryonic day (E) 10.5 to term. We found that each radial artery carrying maternal blood into the uterus branched into 5–10 dilated spiral arteries located within the metrial triangle, populated by uterine natural killer (uNK) cells, and the decidua basalis. The endothelial-lined spiral arteries converged together at the trophoblast giant cell layer and emptied into a few straight, trophoblast-lined “canals” that carried maternal blood to the base of the placenta. Maternal blood then percolated back through the intervillous space of the labyrinth toward the maternal side of the placenta in a direction that is countercurrent to the direction of the fetal capillary blood flow. Trophoblast cells were found invading the uterus in two patterns. Large cells that expressed the trophoblast giant cell-specific gene Plf (encoding Proliferin) invaded during the early postimplantation period in a pattern tightly associated with spiral arteries. These peri/endovascular trophoblast were detected only 150–300 μm upstream of the main giant cell layer. A second type of widespread interstitial invasion in the decidua basalis by glycogen trophoblast cells was detected after E12.5. These cells did not express Plf, but rather expressed the spongiotrophoblast-specific gene Tpbp. Dilation of the spiral arteries was obvious between E10.5 and E14.5 and was associated with a lack of elastic lamina and smooth muscle cells. These features were apparent even in the metrial triangle, a site far away from the invading trophoblast cells. By contrast, the transition from endothelium-lined artery to trophoblast-lined (hemochorial) blood space was associated with trophoblast giant cells. Moreover, the shaping of the maternal blood spaces within the labyrinth was dependent on chorioallantoic morphogenesis and therefore disrupted in Gcm1 mutants. These studies provide important insights into how the fetoplacental unit interacts with the maternal intraterine vascular system during pregnancy in mice. © 2002 Elsevier Science (USA)

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development of the embryo and fetus and to sustain normal pregnancy-related changes in maternal physiological systems. In mice, genetic evidence has shown that uterine natural killer (uNK) cells regulate at least part of the vasodilation that occurs during gestation to increase maternal blood flow to the implantation site. Several indirect lines of evidence suggest that the changes in the maternal blood space are also directed by the implanted conceptus. First, some complications of human pregnancy are associated with defective maternal vascular adaptation to pregnancy (Croy et al., 2000). Preeclampsia, a common and serious third-trimester complication of pregnancy in humans, is believed to result in part from inadequate maternal blood flow to the implantation site, an event associated with defective trophoblast invasion during the first trimester (Roberts and Cooper, 2001). Abnormal placentation is also associated with miscarriage and fetal growth restriction (Jackson et al., 1995; Macara et al., 1995), other common and serious clinical conditions of human pregnancy. Second, although artificial induction of decidualization results in uterine stromal cell changes similar to those of normal pregnancy, the vasculature does not develop normally (Rogers and Gannon, 1981, 1983; Rogers et al., 1982). Third, cells of the rodent placenta, particularly trophoblast giant cells, express several factors with potent angiogenic (e.g., VEGF, Proliferin) and vasoactive (e.g., nitric oxide, Adrenomedullin) effects (Cross et al., 2002). Despite these lines of evidence, the precise molecular mechanisms that regulate maternal vascular development during gestation and its relationship to fetoplacental development are largely unknown.

While some of the gross anatomy and physiology of the mouse and human placenta are different, they share considerable cellular and molecular features (Hemberger and Cross, 2001; Rossant and Cross, 2001). Nonetheless, understanding the interactions of the fetoplacental unit with the uterine vasculature at a molecular level, using the mouse as a model system, has been hampered by an incomplete understanding of the normal anatomy of the placental circulation and of the spatial and temporal relationships between placental trophoblast cells and the vasculature. In addition, tools and approaches for being able to analyze the vasculature in mutant mice have not been widely available. We approached these problems by preparing three-dimensional, plastic vascular casts and analyzing the cell types localized in and near the circulation by marker analysis on histological sections. The results suggest that, although trophoblast cells invade the uterus and arteries supplying the implantation site are dilated and amuscular, these two events are spatially and/or temporally discrete in mice. The transition to the hemochorial blood space and the subsequent shaping of the intervillous maternal blood spaces of the labyrinth are, by contrast, directly controlled by placental trophoblast cells.

**MATERIALS AND METHODS**

**Mice.** Experimental procedures were approved by the Animal Care Committee of Mount Sinai Hospital and conducted in accord with guidelines established by the Canadian Council on Animal Care. CD-1 (ICR) mice were purchased from Harland Sprague-Dawley (Indianapolis, IN) and housed conventionally. The morning that a vaginal copulation plug was detected was designated embryonic day (E) 0.5. Experiments were performed between E10.5 and E17.5, where E18.5 is full term. Gcm1 mutant mice were bred and conceptuses were PCR genotyped as previously described (Anson-Cartwright et al., 2000).

**Histology.** Histological analysis was performed on at least three different placentas per stage of development (range of 3–10), obtained from four to five different mice. Placentas for immunofixation were collected from mice that were killed by cervical dislocation. The uteri were removed and cut between implantation sites. The antimesometrial uterine muscle and membranes of each segment were cut open to facilitate immunofixation in 4% paraformaldehyde. In other cases, the uterine and ovarian vessels were tied before the uteri were removed to maintain the blood cells in the vascular space. The uteri were immersed en bloc in 4% paraformaldehyde overnight and dehydrated with gradient alcohol before being divided into individual implantation sites. In some cases, placentas were fixed by perfusion fixation and the vasculature filled with India ink to outline the maternal vascular spaces. For these experiments, the procedure for perfusion described below was used to clear blood from the lower body vasculature of the pregnant mouse, and then 4% paraformaldehyde was infused for 10 min, followed by infusion with India ink. The abdomen was then opened, the uterine and ovarian vessels tied to contain the ink, and the uterus was removed en bloc and immersed in 4% paraformaldehyde for 24 h. Tissues were dehydrated in alcohol, embedded in paraffin blocks, and cut into 5-µm sections. Samples were processed for transmission electron microscopy after immersion fixation in glutaraldehyde.

Serial sections were stained with hematoxylin and eosin (H&E) for general morphology, elastin stain (Sigma Accustain; Sigma Chemical, St. Louis, MO), Masson trichrome (Sigma), and penta-chrome (Garvey et al., 1986) to identify elastin, smooth muscle, and collagen fibers in the vascular wall, and PAS (EM Science, Gibbstown, NJ) to identify uNK cells. Additional sections were processed by immunohistochemistry to label cytokeratin (DAKO Diagnostics Canada, Mississauga, Canada; Z0622, pAb, rabbit anti-Keratin, WSS, 1:1200) of trophoblast cells, smooth muscle actin (DAKO, M0851, mAb, anti-human smooth muscle actin, 1:50) of vascular smooth muscle cells, and Factor VIII antigen (DAKO, A0082, pAb, rabbit anti-human von Willebrand Factor, 1:800) of endothelial cells. Keratin and Factor VIII antigens were retrieved by trypsin incubation, and smooth muscle actin antigen was retrieved by microwave heating in 10 mM citrate buffer. The standard ABC method was applied with a Vector ABC Staining Kit. Special procedures to block the endogenous mouse immunoglobins when using the mouse monoclonal antibody to identify mouse smooth muscle cells were applied with the DAKO ARK (DAKO, K3954). Histochemical staining for alkaline phosphatase activity was performed by incubating dewaxed sections in NBT/BICP substrate (Life Technologies, Rockville, MD; Gibco/BRL BCIP/NBT Combo, Cat. No. 18280-016) for 4 h at room temperature in the dark. Slides were counterstained by using nuclear fast red. In situ hybridization with digoxigenin-labeled riboprobes for Tpbp

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buffered saline (PBS) so that the clearing and vena cava. The uterus was exposed in a bath of warm phosphate-
inserting an additional catheter in the intra-abdominal inferior thoracic aorta as described above, then opening the abdomen and 2, 11 and 13 placentas) were prepared by inserting a catheter in the retrogradely into the inferior vena cava catheter (blue pigment catheter (red pigment added; Batson et al.) was introduced into the descending thoracic aorta. A cut was made in the right atrium to serve as an exit point for perfusion, and a loose tie was placed around the intrathoracic inferior vena cava. An infusion pump was used to perfuse the lower body vasculature at 4 ml/min with 10–20 ml of warm (40–45°C) heparinized xylocaine (1% xylocaine in 0.9% NaCl with 1 IU heparin/ml) to dilate the vasculature and clear it of blood, followed by 10 ml of the same perfusate chilled to 4°C. Chilling the tissues prolonged the setting time and also hardened the vessels. The prechilled components of a methyl methacrylate casting compound (Polysciences Inc., Warrington, PA; Batson’s number 17; 5 ml monomer base, 1.5 ml catalyst, 0.1 ml promoter) were mixed and their viscosity reduced by adding chilled methyl methacrylate monomer (2.4 ml; Jet Liquid, Lang Dental, Wheeling, IL). The liquid plastic mixture was infused at 0.4 ml/min for 2 min and then 0.7 ml/min until a total of 4–5 ml casting compound had been infused. In 6 experiments, femoral arterial pressure was monitored. Peak pressures occurred during the infusion of the plastic mixture but remained below typical systolic blood pressures for mice (peak pressure averaged 80 mm Hg, range of 45–110 mm Hg). After infusion of the casting compound, the ligature around the inferior vena cava was pulled tight, and the infusion syringe was pressurized to ~20 mm Hg to sustain vessel inflation while the plastic was setting. After the casting compound had hardened, the uterus was removed and transected between implantation sites. Each segment was immersed in 20% KOH to digest the tissue. Casts were washed in water and air-dried before examination under a stereomicroscope or were sputter-coated with gold for scanning electron microscopy. Some casts were dissected to reveal inner structures. Between 3 and 16 casts of the maternal vasculature were examined from several pregnant mice at different gestational ages (E10.5, n = 1, 15 placentas; E11.5, n = 1, 16 placentas; E12.5, n = 1, 12 placentas; E13.5, n = 1, 13 placentas; E14.5, n = 5, 3–10 placentas each; E15.5, n = 1, 13 placentas; E17.5, n = 1, 12 placentas). For implantation sites of Gcm1 mutants, part of the fetus was removed for DNA analysis before tissue digestion.

Two-color casts of the maternal placental circulation (E14.5, n = 2, 11 and 13 placentas) were prepared by inserting a catheter in the thoracic aorta as described above, then opening the abdomen and inserting an additional catheter in the intra-abdominal inferior vena cava. The uterus was exposed in a bath of warm phosphate-buffered saline (PBS) so that the clearing and filling of the uterine vasculature could be monitored. After clearing blood from the vasculature with warm perfusate as described above, a dual infusion pump was used to simultaneously infuse plastic into the aorta catheter (red pigment added; Batson’s #17, Polysciences Inc.) and retrogradely into the inferior vena cava catheter (blue pigment added; Batson’s #17, Polysciences Inc.) until both colors were observed entering the intrauterine vasculature.

For fetal side casts at E12.5, E13.5, E14.5, E15.5, and E17.5, pregnant mice (n = 1–5 for each stage; 2–10 placentas cast per mouse) were killed by cervical dislocation, and the uterus was rapidly removed en bloc and immersed in ice-cold PBS (Smith, 2000). An implantation site was cut from the uterus and placed in a petri dish under a surgical microscope. The uterine muscle was cut along the antimesometrial edge to expose the yolk sac. The yolk sac and amniotic membranes were then cut near the placenta to expose the embryo and the placental surface. The petri dish was placed under a heat lamp, and the embryo was further warmed and wetted with 40°C PBS until the heart began to beat and the umbilical vessels dilated (Smith, 2000). The umbilical vessels could be distinguished because the artery had a prominent pulse and, when flow first began, the vein had a bright red color. A few drops of 3% paraformaldehyde were applied to the umbilical vessels to reduce vasospasm, and then a small cut was made in both umbilical vessels. A tapered glass cannula was broken with forceps so the tip was the appropriate diameter. A double-lumen catheter was attached to the cannula. One lumen was primed with a warmed xylocaine/heparin solution and the other with casting compound. A trace of color was added to the casting compound so that flow could be visualized. The cannula tip was advanced into one umbilical vessel and the other vessel served as the exit point. Xylocaine/heparin to clear the blood followed by the plastic were infused by hand. Because of the small diameter of the vessels and the viscosity of the plastic, casts of the fetal circulation were only made at E12.5 and later.

RESULTS

Overview of maternal circulation through the placenta. After implantation, the pregnant uterus undergoes a radical transformation in structure. Uterine stromal cells proliferate and differentiate—a process called decidualization—resulting in a uterine wall that is about fivefold thicker than in the nonpregnant state. New blood vessels develop within the decidua, eventually allowing maternal blood to reach the implantation site. uNK cells traffic first to the implantation site and, by E10.5, are localized to the metrial triangle (Fig. 1A). By E10.5, the placenta has a mature structure and consists of a layer of trophoblast giant cells in contact with the decidua basalis, an underlying cell dense region called the spongiotrophoblast, and the inner labyrinth where nutrient transfer occurs between the maternal and fetal blood spaces (Fig. 1A).

To understand how maternal blood gets into and out of the placenta, we made vascular casts by injecting liquid plastic into the large vessels leading to and from the uterus. We found that the overall structure of the casts was similar on all days examined between E10.5 and E17.5. The maternal arterial supply to the placenta originated from radial arteries that entered the uterus through the myometrium on the mesometrial side of the uterus. Branches of the radial artery either passed laterally in the myometrium or traversed the myometrium and entered the metrial triangle (Fig. 1). The supply arteries then branched into several (5–10) spiral-shaped arteries (Figs. 1D and 1F). After traversing the decidua basalis, the spiral arteries converged to form a small number (between 1 and 4) of centrally located arterial canals. Comprehensive histological analysis revealed that the convergence point of the spiral arteries was at the trophoblast giant cell layer (Fig. 1C). Immunostaining
showed that the central canals were lined by cytokeratin-positive (i.e., trophoblast) cells (Fig. 1C), as was all of the maternal blood space within the labyrinth and the venous channels in the spongiotrophoblast (see below). The arterial canals had extremely large diameters (300–600 μm) and coursed to the base of the placenta before leading into the small tortuous trophoblast-lined sinusoid spaces of the labyrinth (Fig. 1F). Maternal blood drained from the labyrinth through venous channels that crossed the spongiotrophoblast and giant cell layers into peripherally located, cup-shaped venous sinuses in the decidua basalis (Figs. 1E and 1F). The venous sinuses were lined by endothelial cells (data not shown) and were prominent in histological sections only when maternal blood vessels were ligated prior to tissue fixation (Fig. 1B). Otherwise, the blood in the venous spaces readily leaked out during tissue processing and the venous sinuses collapsed. The venous sinuses drained into wide, flattened veins (Fig. 1E), which progressively merged as they traversed the outer region of the metrial triangle and exited into the radial veins outside the myometrium.

**Structure of the spiral arteries within the metrial gland and decidual regions.** From E10.5 to term, the placenta undergoes tremendous growth due to expansion of the labyrinth layer. During this interval, the vascular casts showed that the spiral arteries and central arterial canals elongated and the trophoblast-lined sinusoids became smaller and more intricate (Fig. 2). Strikingly, while their number did not change, the diameter of the spiral arteries based on measurements of the casts increased from ~60 μm...
at E10.5 to ~150 μm at E14.5 (Fig. 2). An increase in spiral artery diameter was also obvious from histological sections, but was not quantitated as tissues shrink considerably during processing. This dramatic change in vessel diameter prompted a more detailed investigation of their structure and the cell types associated with them.

Curiously, the spiral arteries often had diameters greater than the radial arteries from which they arose (Figs. 2C and 3A). Close examination of the casts showed circumferential constrictions around the radial arteries that were presumably associated with sphincters (Fig. 3A). These sphincters were not apparent in spiral arteries, however. In the metrial triangle, spiral arteries supplied a dense microcirculation particularly between E11.5 and E14.5 (Fig. 3B). The diameter of the spiral arteries appeared to be relatively consistent throughout their length until the junction with the central canals. Interestingly, though, the appearance of the cast surface changed during its course, reflecting a change in the cells lining the lumen of the vessel. Whereas the surface of the spiral artery casts had densely packed, small, but prominent indentations reflecting the “imprints” left by endothelial cells, the casts of trophoblast-lined central canals had a much smoother appearance. The small, endothelial cell-sized impressions were replaced by flatter and larger cell imprints. This transition in the luminal surface was obvious ~150–300 μm upstream of the spiral artery to central canal junction (Figs. 3C and 3D).

**Endovascular and interstitial routes of trophoblast cell invasion into the uterus.** To investigate whether the cells lining the lower segment of the spiral arteries were trophoblast cells, we performed cytokeratin immunostaining on serial histological sections of implantation sites at different stages of gestation. Prior to E12.5, the border of the cytokeratin-positive cells was defined by the trophoblast...
giant cell layer and there was no evidence for bulk invasion of the uterus by trophoblast cells. Close inspection of histological sections that contained segments of spiral arteries revealed that cytokeratin-positive trophoblast cells were present around and within the lumen of maternal vessels (Figs. 4C and 4D). The spiraling nature, central location, and diameter of the vessels were consistent with them being spiral arteries, although they lacked obvious smooth muscle (see below). The peri/endovascular trophoblast cells were only detected at most 150–300 μm from the main layer of trophoblast giant cells (Fig. 4B). In addition, at these early stages, the trophoblast cells that had invaded beyond the main giant cell layer were tightly localized around the arteries. They showed no general invasion of the interstitium in the decidua (Figs. 4B and 4E) and did not invade the venous sinuses (data not shown).

After E12.5, another type of trophoblast cell appeared in the decidua outside the trophoblast giant layer, called glycogen trophoblast cells. These cells were also cytokeratin-positive but had a "foamy" appearance in sections due to loss of cytoplasmic contents during tissue processing (Fig. 5). Unlike the peri/endovascular migration observed at earlier stages, the glycogen trophoblast cells had a diffuse, interstitial invasive pattern and did not preferentially localize to vessels. In fact, even in regions close to spiral arteries, the glycogen trophoblast cells did not approach or invade the vessel wall (Figs. 5B and 5C). At later stages, glycogen trophoblast cells were also found as far as the distal regions.

**FIG. 3.** Special features of the maternal arterial supply to the mouse placenta. (A) Radial arteries. Circumferential bands of vasculature and localized sites of constriction (arrows) suggest the presence of sphincters around radial arteries at E14.5. (B) Spiral arteries in the metrial triangle. Discrete regions of dense microcirculation were observed in the metrial triangle region (arrow). These structures were most prominent between E11.5 and E14.5. Microcirculation arose directly from the spiral arteries with no intermediate-sized connecting vessels. (C) Spiral arteries within the decidua basalis converging to form a central arterial canal. (D) A high magnification view of the cast surface from (C). Note the smooth surface associated with the imprint of the trophoblast cells lining the arterial canals and the prominent nuclear indentations associated with endothelial cells lining the spiral arteries (arrow defines transition between the two types of cast surface). Spiral arteries adjacent to canals were smooth, suggesting they were trophoblast lined. C, central arterial canal. Bars, 300 μm.
of the decidua basalis, in places beyond the limit of the peri/endovascular trophoblast cell invasion (Fig. 5C).

To determine whether the endovascular and interstitial trophoblast cells invading the uterus could be distinguished by using markers, we first used periodic acid Schiff (PAS) staining to detect glycogen trophoblast cells. PAS-positive, cytokeratin-positive trophoblast cells were present throughout the interstitium of the decidua basalis only at E12.5 and later. The artery-associated trophoblast cells were PAS-negative. PAS-staining extended back into the spongiotrophoblast layer and the glycogen trophoblast cells expressed the mRNA for Tpbp (also

FIG. 4. Peri- and endovascular invasion of spiral arteries by trophoblast giant cells. (A–D) Histological sections from E12.5 implantation sites immunostained for cytokeratin shown at low (A), intermediate (B), and two examples at high (C, D) magnification. (E, F) Serial histological sections at E12.5 shown at low magnification stained for cytokeratin (E) and PAS (F). Note that the cytokeratin-positive cells (brown) do not stain with PAS (pink). (G, H) In situ hybridization for Plf mRNA. High magnification view of peri/endovascular cells in two different spiral arteries. Arrow, peri/endovascular trophoblast cells; arrowheads, spiral artery without trophoblast cells.

FIG. 5. Interstitial invasion of the decidua by glycogen trophoblast cells after E12.5. (A–C) Histological sections from E14.5 implantation sites immunostained for cytokeratin shown at low (A) and intermediate (B, C) magnification. Bracket shows region of cells invading into the interstitium of the decidua. Dashed line indicates position of trophoblast giant cell layer. The area shown in (C) is located further from the placenta compared with (B), and shows a spiral artery without peri/endovascular trophoblast cells. (D–H) Serial histological sections at E14.5 shown at low magnification stained for cytokeratin (D) and PAS (E). Note that the cytokeratin-positive cells outside the trophoblast giant cell layer (brown) also stain with PAS (pink). (F) In situ hybridization for Tpbp mRNA. Sp, spongiotrophoblast. Arrow, peri/endovascular trophoblast cells; arrowheads, spiral artery without trophoblast cells; dotted line, trophoblast giant cell layer. 

FIG. 6. Histological characteristics of the vessel wall of radial artery and spiral arteries shown at different levels at E13.5. Serial cross-sections of implantation sites at each level were immunostained for factor VIII, smooth muscle actin and cytokeratin, or stained with PAS stain. Arrow, uNK cell with intense PAS-positive granules.
called 4311), a spongiotrophoblast-specific gene (Fig. 5F), consistent with the hypothesis that glycogen trophoblast cells are derived from spongiotrophoblast. Peri/endothelial mRNA (data not shown), but they did express mRNA for Plf (encoding Proliferin), a trophoblast giant cell-specific gene (Figs. 4G and 4H).

Spiral arteries are dilated and lack smooth muscle far upstream of the most deeply invaded trophoblast cells. The discovery of peri/endothelial trophoblast cells in spiral arteries that appeared to lack muscular walls was striking. To confirm the absence of smooth muscle, and to describe precisely where it occurred along the length of the spiral artery, we analyzed cross-sections of arteries throughout the thickness of the uterine wall using immunocytochemistry, perichrome, and elastic stain to visualize the artery wall and to detect different cell types. The radial arteries in the mesometrial side of the myometrial region had a typical arterial structure, including a thin, factor VIII-positive endothelium, smooth muscle actin-positive media, and an inner elastic lamina (Fig. 6). However, the spiral arteries in the metrial triangle region, although lined by endothelium, had sparse elastin (data not shown) and a thin, incomplete smooth muscle layer (Fig. 6). Further downstream into the distal decidual region, no smooth muscle actin-positive cells were detected surrounding the arteries. The transition from maternal endothelium- to trophoblast-lined blood space occurred only further downstream, in the decidual basalis proximal to the placenta (Fig. 6). Therefore, the loss/absence of smooth muscle surrounding the spiral arteries was apparent at a considerable distance (at least 2 mm at E13.5) from where peri/endothelial and interstitial trophoblast cells were detected. In contrast, the absence of smooth muscle in the spiral arteries was first apparent in the metrial triangle, a region rich in uNK cells that contain prominent PAS-positive granules (Fig. 6).

Structure and formation of the maternal blood space within the labyrinth. From the trophoblast giant cell border inwards, the maternal blood space is lined by trophoblast cells. The arterial canals traverse the spongiotrophoblast and lead to the base (embryonic side) of the labyrinth layer before leading into smaller trophoblast-lined canal branches (Fig. 7). The canal branches often formed a ring-like space (Fig. 7A) near where the visceral yolk sac membranes attached to the placenta. The large canal branches lead abruptly into fine (~47 μm at E10.5, 17 μm at E15.5), irregularly shaped, and highly anastomosing sinuoids that extended radially outwards as well as back toward the spongiotrophoblast layer. The sinuoids converged at the spongiotrophoblast border to form larger venous channels that passed through the spongiotrophoblast and giant cell layer to lead into the venous sinuses in the decidua basalis. The small maternal blood spaces within the labyrinth are lined not by endothelium but rather by specialized trophoblast subtypes. Within the labyrinth, three layers of trophoblast cells separate the maternal blood space from the fetoplacental endothelium; a discontinuous layer of cuboidal, mononuclear trophoblast cells line the lumen of the sinuoids and is underlain by two layers of elongated and multinucleated syncytiotrophoblast (Fig. 7E). The two sycytiotrophoblast layers are distinct at an ultrastructural level (Fig. 7E). In addition, the trophoblast layer that is closest to the maternal blood space has intrinsic alkaline phosphatase activity (Fig. 7F).

The fact that the maternal blood space from the trophoblast giant cell layer inwards is lined by trophoblast cells implies that it is shaped by morphogenesis of trophoblast layers. This idea is supported by the fact that, as the chorioallantoic interface undergoes more extensive branching during development, the trophoblast-lined sinuoidal spaces become progressively smaller (Fig. 2). To test this directly, we examined the maternal blood spaces in the placentas of Gcm1 mutant conceptuses. Gcm1 is expressed in a subset of chorionic trophoblast cells, and that defines where branch points will occur (Anson-Cartwright et al., 2000). Gcm1-deficient mutants fail to make a placental labyrinth layer due a failure in chorioallantoic branching (Anson-Cartwright et al., 2000). Histological sections and vascular casts showed that maternal blood is able to penetrate through the trophoblast giant cell and spongiotrophoblast layer to reach the chorioallantoic interface in Gcm1 mutants (Fig. 8). However, the terminal sinuoidal spaces remain much larger that normal (Figs. 8C and 8E vs 8D and 8F).

**Fetal circulation through the placenta.** The anatomy of the maternal blood space in the labyrinth suggested that the most oxygenated blood is brought first to the fetal side of...
the placenta and courses retrograde back toward the maternal side. To visualize the fetoplacental circulation and compare its relationship with the maternal circulation, we prepared casts of the fetal circulation by injecting casting compound into umbilical vessels (Figs. 9E–9G). In addition, we prepared dual vascular casts in which a blue plastic was injected on the maternal side and a red plastic was injected on the umbilical side (Figs. 9A–9D). We found that a single umbilical artery branched extensively within the central region of the placenta (Figs. 9C and 9D) and the arterioles then traveled into the labyrinth. The density of blood spaces within the labyrinth was strikingly shown by the fetal vascular casts, particularly when considered along with the density of the maternal blood space. The dominance of blood space within the labyrinth is obscured in most histological sections because blood spaces tend to collapse when the tissue is not perfusion fixed. From E12.5 to term, the diameter of the umbilical vessels increased from ~300 to 500\(\mu\)m, and the labyrinth layer increased in both diameter (from ~ 6 to 8 mm) and thickness (from ~2 to 3 mm), but the diameter of the fetal capillaries (~15 \(\mu\)m) was unchanged.

To examine the pattern of arteriolar and capillary branching within the placenta, we examined a series of fetal-side casts in which the capillary network was incompletely filled either from the arterial or the venous side (Figs. 9F and 9G). Casts from the arterial side showed that the fetoplacental arterioles extended radially into the labyrinthine layer and traveled largely unbranched toward the spongiotrophoblast side (Fig. 9F). The arterioles were lined by Factor VIII-positive endothelial cells (Fig. 9H) and had smooth muscle actin-positive cells in their walls from E10.5 to term (Fig. 9I). Upon reaching the spongiotrophoblast border, the arterioles branched abruptly into a dense bed of amuscular capillaries that enclosed the arterioles as they extended back toward the fetal side of the placenta (Fig. 9F). Therefore, fetal blood flow through the capillaries of the labyrinth runs counter to that of maternal blood. Viewed from the maternal side, the capillary network surrounding an arteriole formed a lobular or cotyledonary structure, each of which was a densely packed villous tree. In contrast to the endothelium of the arterioles, the endothelium of the fetal capillaries was Factor VIII-negative (Fig. 9). The placental venules which collected the capillary blood from the fetal side of the labyrinth were relatively short and superficial and ultimately drained into a single umbilical vein (Fig. 9G).

**DISCUSSION**

These studies provide the first comprehensive description of the maternal and fetal blood circulation through the mature placenta in mice. In addition to defining the normal anatomy, the work gives insights into the nature of maternal–fetal interactions that occur during pregnancy. First, by studying the various cell types that lie in association with the vascular spaces, we have been able to give insights into the potential role of trophoblast cell invasion and its importance for maternal vascular adaptations to pregnancy. We found that, while mouse trophoblast cells do invade the uterus in ways analogous to invasive extravillous cytotrophoblast cells in humans, the vascular remodeling that results in dilation of the spiral arteries occurs independently of invading trophoblast cells. Our data do not rule out, however, an indirect role of trophoblast cells in promoting maternal vascular remodeling, such as effects of a soluble vasoactive factor. Second, by identifying specific markers for them, we were able to define the trophoblast cell subtypes and their likely embryological origins, associated with different areas of the maternal blood space. One important finding is that two types of invasion into the uterus occur—peri-endovascular and interstitial—and that they are due to distinct trophoblast cell subtypes. Finally, by studying development in both wild type and Gcm1 mutant conceptuses, we found that shaping of the hemochorial maternal blood space within the placenta is determined by morphogenesis of the trophoblast cells. These studies therefore provide insights that are essential for understanding maternal–fetal interactions during gestation. In addition, the markers and analytical approaches that we have developed for our studies will be of clear use for studying mouse mutants in which defects in placental vascular development or trophoblast–vascular interactions are suspected.

**Anatomy of the maternal and fetal blood circulations through the placenta in mice.** The combination of vascular casts and detailed histological analysis allowed us to describe the complete pattern of both maternal and fetal blood flow through the mature mouse placenta (Fig. 10). Maternal blood enters the uterus through radial arteries that branch into 5–10 spiral arteries. The spiral arteries become dilated in the metrial triangle, and from that point downstream, they progressively lose elastin and smooth muscle. They then converge together at the trophoblast giant cell layer and, at that point, are no longer conventional vessels in that the blood space is lined by trophoblast cells. From the giant cell layer inwards, blood is carried via straight canals that pass to the base (fetal side) of the placenta. Although we observed between 1 and 4 central arterial canals, others have previously described a single structure in mice (Redline and Lu, 1989). This difference may be due to strain differences in the number of canals, or the presence of multiple canals may have been overlooked as the earlier study was based on histology alone. After passing through the arterial canals, maternal blood then percolates back to the apical (maternal) side of the placenta through tortuous, anastomosing, trophoblast-lined sinuses in the labyrinth. The sinuses coalesce into larger channels that traverse the spongiotrophoblast and giant cell layers and lead into maternal endothelial cell-lined, large venous sinuses in the decidua basalis. Fetal blood passes through the labyrinth in the opposite direction to that of the maternal blood in that fetoplacental arterioles pass to...
The spongiotrophoblast (apical) side of the labyrinth before branching into capillaries that course back toward the base. The structure of the blood vessels on the maternal and fetal sides gives interesting insights into the potential for physiological control of blood flow. On the maternal side, the muscularized wall and sphincters of the radial artery may control blood flow to the implantation site, whereas such control by the largely amuscular spiral arteries is unlikely. It is unknown whether the amuscular arteries have the capacity to dilate further, perhaps by additional proliferation of endothelial cells, but the fact that the spiral arteries increase in diameter from E10.5 to E17.5 is certainly consistent with this. On the fetal side, we observed that the small fetoplacental arterioles have smooth muscle layers and therefore may play a role in regulating local fetoplacental perfusion to match perfusion and/or oxygenation of adjacent maternally perfused sinusoids. In vitro evidence supports such a function in the fetoplacental vasculature of the human placenta (Howard et al., 1987).

As in many other hemochorial placentas, the overall circulation through the mouse placenta is consistent with countercurrent exchange (Dantzer et al., 1988; Wooding and Flint, 1994). Oxygen-poor blood from the fetus is carried in arterioles that course directly to the maternal side.
of the placental labyrinth before branching into capillaries that course back toward the fetus. In contrast, oxygen-rich maternal blood is delivered via the central canals directly to the base (fetal side) of the labyrinth. Fetal and maternal blood then travel through the microcirculation within the labyrinth in opposite directions. In this way, fetal venous blood exiting from the capillaries would equilibrate with maternal arterial blood entering the trophoblast-lined sinusoids (Fig. 10). This system would thereby maximize the umbilical venous oxygen delivery to the fetus.

The countercurrent arrangement of the maternal and fetal blood spaces suggests intricate regulation of their development. With respect to the fetal side, it means that the points where the arterioles branch into capillaries must be regulated by proximity to the apical edge (adjacent to the maternal side) of the placenta. Indirect evidence suggests that factors from the trophoblast cells in the labyrinth regulate fetoplacental vascular development (Rossant and Cross, 2001). The formation of the maternal blood space in the labyrinth is interesting given that it is lined by trophoblast cells. The sinusoid spaces are formed as branches of the chorioallantoic interface push into the large amorphous space. As such, the complexity of the maternal blood space simply reflects the complexity of the outer surface of the chorioallantoic villi. The spaces remain large if the chorioallantoic interface fails to branch, as we observed in Gcm1 mutants. The development of the central canals that bring maternal blood down to the base of the labyrinth is intriguing because their structure implies that the trophoblast surface becomes highly organized and coalesces into one or only a few substantial tubes. How sites of canal formation are selected and what promotes the presumed convergence of several amorphous spaces, that are apparent at early postimplantation stages (unpublished data), into one or only a few straight canals are unknown.

Comparison of the mouse and human placental circulation. In general, the circulatory anatomy of the mouse placenta appears similar to that described previously for placentas of other rodents (Wooding and Flint, 1994). In addition, it appears that the circulation through the mouse placenta is extremely similar to that of humans. On the maternal side of the placenta in both species, maternal blood passes through dilated, amuscular spiral arteries before entering a space created by the conceptus that is densely filled with fetal placental villi. In humans, maternal blood emerges from spiral arteries into a trophoblast-lined intervillous space that surrounds the complex, highly branched villous tree. Many intervillous channels are tortuous and of capillary dimensions (Burton, 1987) and thus are similar to the trophoblast-lined sinusoids of the mouse, usually described as a labyrinth. How blood gets into the intervillous space may be slightly different between species. In mice, maternal blood is confined to a few trophoblast-lined arterial canals that direct blood to the basal side of the placenta, whereupon it returns toward the maternal side through a dense network of sinusoids. By contrast, canal-like structures have not been described in humans. On the fetal side of the placenta in both species, umbilical vessels branch near the center of the chorionic plate to supply the placenta. In mice, the placental arterioles penetrate to the distal surface of the placenta and then branch abruptly into capillaries that return centrally surrounding the arterioles. This arrangement is similar to the “paravascular networks” surrounding larger villous vessels in the human placenta (Burton, 1987).

The many obvious similarities between the murine and human placental circulations emphasize the utility of the mouse as a model for understanding human placentation. In addition though, our studies have highlighted some features of the mouse placental circulation, particularly the interactions of invasive trophoblast cells with maternal spiral arteries, that would at first glance appear at odds with the current descriptions of human placentation (see below). While they could reflect real species differences, it is equally possible that our understanding of human placental development is incomplete.

Trophoblast cell invasion does not directly influence spiral artery wall structure in mice. Modification of spiral artery structure, including vessel dilation and loss of smooth muscle cells and elastin, is thought, in humans, to be due to the actions of cytrophoblast cells invading and degrading the walls of the spiral arteries (Cross, 1996). It is true, however, that the evidence in support of this idea is only correlative, and indeed some studies suggest that spiral artery dilation occurs prior to the vessels being invaded (Craven et al., 1998). Because of the small size and short gestation of mice, we were able to observe spiral arteries throughout their entire length in the uterine wall and at multiple time points during gestation. We observed dilated and amuscular spiral arteries in mice in the metrial triangle and decidua, similar to those described in humans. As this region is largely devoid of trophoblast cells before E12.5, the atypical arterial structure could not obviously be attributed to the direct effects of trophoblast cell invasion. Trophoblast giant cells do express adrenomedullin and nitric oxide, potential vasodilators (Cross et al., 2002), and it is possible that these factors could affect the spiral arteries. Notably, however, they would have to act at a considerable distance.

In contrast, uNK cells have been shown to be important in spiral artery adaptations to pregnancy in mice, as shown by the fact thatNK cell-deficient mice have smaller diameter and muscularized arteries within the decidua region (Croy et al., 2000). uNK cells are present in the decidua and metrial triangle from early postimplantation stages and peak in numbers between E10.5 and E14.5 in mice. They are also abundant in the human uterus during pregnancy. Thus, as in mice, uNK cells could mediate arterial dilation of decidual arteries in early human pregnancy when such changes occur in the absence of cellular interactions with extravillous cytrophoblasts (Craven et al., 1998).

Two modes of trophoblast invasion by distinct cell types. Distinct modes of trophoblast invasion were observed in mice that are reminiscent of those described in humans. We observed early but limited trophoblast inva-
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Plf the two cell types. Specification gives insights into the likely embryological origins of molecular marker studies would resolve this issue.

in mice is unclear given that the order of endovascular and invasion of the decidua. Moreover the PAS-negative cells showed uniform cytokeratin immunoreactivity were not present in the interstitium and were tightly associated with the spiral arteries. These distinct cell behaviors were distinguishable by differences in PAS staining as well as in molecular marker expression. The specific marker expression gives insights into the likely embryological origins of the two cell types. Specifically, peri/endovascular trophoblast cells expressed the Plf gene, a specific marker of trophoblast giant cells (Linzer et al., 1985; Ma et al., 1997), and not Tpbp, a spongiotrophoblast-specific gene (Lescisin et al., 1988). Others have reported that the peri/endovascular trophoblast cells also express the Prlpl and Prlpm genes (encoding PLP-L and PLP-M) (Toft and Linzer, 2000). Like Plf, Prlpl and Prlpm are exclusively expressed in trophoblast giant cells within the placenta. The peri/endovascular cells do not express all giant cell-specific genes such as Pl1 (M.H. and J.C.C., unpublished observations). Therefore, we conclude that peri/endovascular trophoblasts are a specialized subtype of trophoblast giant cell. It is important to note that the peri/endovascular trophoblast layer is contiguous with the main trophoblast giant cell layer surrounding the ectoplacental cone/spongiotrophoblast layer. Therefore, it is likely that the peri/endovascular trophoblast giant cells have differentiated as part of the giant cell lineage as opposed to adopting a giant cell fate after reaching their location. The interstitial invading cells are what have been previously called glycogen trophoblast cells. The fact that these cells express the Tpbp gene, a spongiotrophoblast-specific gene, and that cells of the same morphology and PAS staining characteristics also appear within the spongiotrophoblast layer implies that glycogen trophoblast cells are a differentiated derivative of spongiotrophoblast cell.

The distinct origin and marker expression of the peri/endovascular and interstitial trophoblast cells is extremely interesting because such distinctions have not been made for the endovascular and interstitial invading cells in the human placenta. It is unknown whether this represents a species difference or simply the fact that markers have not been found in humans that can distinguish the two cell types. It is an important issue to address though, with respect to understanding the trophoblast defects in preeclampsia, as it is possible that only one cell type or behavior is affected in this disease.

Trophoblast giant cells as regulators of maternal angiogenesis and the hemochorial transition in mice. Trophoblast giant cells in mice have other types of interactions with the maternal vasculature that have not been addressed in humans. For example, trophoblast giant cells produce the angiogenic factors VEGF (Achen et al., 1997; Voss et al., 2000) and Proliferin (Jackson et al., 1994). While there is no direct evidence that these factors specifically target the maternal vasculature to promote ingrowth of new vessels to the implantation site, circumstantial evidence suggests this is their likely function. VEGF and Proliferin are expressed by giant cells, even from early postimplantation stages, and therefore several days before the fetoplacental vasculature even begins to form. Compellingly, the maternal vasculature fails to develop correctly in the decidual region surrounding conceptuses that are deficient for the Gata2/3 transcription factors (Ma et al., 1997). Gata factors have been implicated in transcriptional regulation of trophoblast giant cell-specific genes (including Plf) and Plf expression is reduced in the mutants. The implication is that reduced angiogenic factor expression from giant cells results in failed maternal vascular development. It would be interesting to determine whether extravillous trophoblast cells in humans also regulate angiogenesis in the uterus in the early postimplantation period.

Trophoblast giant cells in mice also play a role in promoting the transition from endothelium-lined artery to trophoblast-lined sinus blood space. This effect can be attributed to the effects of trophoblast giant cells and not simply some unusual aspect of the maternal vascular bed, because hemochorial blood flow is also observed if trophoblast giant cells appear in ectopic sites (Avery and Hunt, 1972; Nozaki et al., 1999). Whether this is due to trophoblast giant cells invading into an artery and replacing the endothelium or whether giant cells have a more subtle way of inducing vascular instability is unknown. Antiangiogenic factors are also expressed in the developing placenta, including soluble Flt1 (He et al., 1999) and Proliferin-related protein (Prp) (Jackson et al., 1994; Linzer and Nathans, 1985), which are specific antagonists of VEGF and Proliferin, respectively. They are expressed by the underlying spongiotrophoblast cells, although Prp is also expressed by giant cells. Expression in the spongiotrophoblast could obviously prevent maternal endothelial cells...
from entering the placenta. The adjacent expression of angiogenic and anti-angiogenic factors could also create the “paradoxical” environment necessary to promote instability of arterial wall structure (Cross et al., 2002).

**Vascular casting and histological analysis as tools for assessment of placental structure.** The vascular casts have vividly illustrated the three-dimensional structure of the blood spaces in the mouse placenta. Vascular casts have been used to visualize maternal circulation through the placenta in other species (Boe, 1950; Leiser et al., 1989; Ogura et al., 1991; Pfarrer et al., 1999; Takenori et al., 1984) and the fetoplacental circulation in rats (Boe, 1950; Lee and Dempsey, 1976) and humans (Burton, 1987; Kaufmann et al., 1988; Leiser et al., 1997). As a caution, though, some problems were frequently encountered, including leakage of plastic, a common problem in the labyrinth of maternal side casts at E10.5. In addition, incomplete filling of the uteroplacental circulation is usually observed in at least some placentas in each mouse. This is likely due to spasm of the muscular sphincters surrounding the radial arteries as these vessels have been reported to be highly vasoactive (D’Angeio and Osol, 1993). The inclusion of the local anesthetic, xylolcaine, in the perfusate enhanced clearance of blood and improved vascular filling, likely by “anesthetizing” the vascular musculature. We do not believe that xylolcaine influences the results in any significant way because it has no significant vasoactive effects. It certainly would not affect the structure of the distal spiral artery and canal, which lack smooth muscle and are thus maximally dilated.

One of the important technical advances from our studies has been the development of histological methods and molecular markers for analyzing the structures of the fetal and maternal circulatory systems through the mouse placenta. The fact that quantitative analysis of the casts can be done for parameters such as vessel number and diameter, means that casting may be a useful approach for studying mouse mutants, which commonly have placentation defects (Hemberger and Cross, 2001; Rossant and Cross, 2001).

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