REPRODUCTION

REVIEW

Uterine natural killer cells: supervisors of vasculature construction in early decidua basalis

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Abstract

Mammalian pregnancy involves tremendous *de novo* maternal vascular construction to adequately support conceptus development. In early mouse decidua basalis (DB), maternal uterine natural killer (uNK) cells oversee this process directing various aspects during the formation of supportive vascular networks. The uNK cells recruited to early implantation site DB secrete numerous factors that act in the construction of early decidual vessels (neoangiogenesis) as well as in the alteration of the structural components of newly developing and existing vessels (pruning and remodeling). Although decidual and placental development sufficient to support live births occur in the absence of normally functioning uNK cells, development and structure of implantation site are optimized through the presence of normally activated uNK cells. Human NK cells are also recruited to early decidua. Gestational complications including recurrent spontaneous abortion, fetal growth restriction, preeclampsia, and preterm labor are linked with the absence of human NK cell activation via paternally inherited conceptus transplantation antigens. This review summarizes the roles that mouse uNK cells normally play in decidual neoangiogenesis and spiral artery remodeling in mouse pregnancy and briefly discusses changes in early developmental angiogenesis due to placental growth factor deficiency.

Reproduction (2015) **149** R91–R102

Introduction

Continuous blood flow to the maternal–fetal interface is vital for healthy pregnancies. Shortly after implantation, decidual neoangiogenesis begins. In humans, capillary growth around the syncytiotrophoblast is reported at 7th–11th day of pregnancy (Zygmunt *et al*. 2003). In mice, primary decidualization and angiogenesis around the embryonic crypt begin at gestation day 5 (GD5), about 12 h after implantation (Tan *et al*. 1999, Cha *et al*. 2012, Croy *et al*. 2012). Growth of these vessels during early endometrial decidualization is followed quickly by vessel linkage, maturation, and pruning. These events occur well in advance of maturation of the hemochorial placenta with opening of the utero-placental circulation occurring about week 12 in humans and about GD9.5–10.5 in the mouse (Hustin & Schaaps 1987, Adamson *et al*. 2002, Aasa *et al*. 2013).

Embedded within the process of normal human and mouse placental development is the physiological modification of the terminal branches from the uterine artery, called spiral arteries (SA). SA remodeling is deemed necessary for enlarging the nutrient-enriched maternal blood supply to the placenta to support the newly developed and rapidly growing fetus. It is also held to make these supplies available on a non-interrupted basis because remodeling makes the arterioles unreactive to vasoactive substances. Impaired decidual vascular development during early angiogenesis, or myometrial SA remodeling, has been linked with pregnancy complications (recurrent spontaneous abortion (RSA; Quenby *et al*. (2009)), preeclampsia (PE; Lyall *et al*. (2013)), and fetal growth restriction (FGR; Williams *et al*. (2009))). Although these are common problems (RSA and PE affect ∼1% of women and ∼3–6% of pregnancies respectively), treatment approaches are limited (Faridi & Agrawal 2011, Ananth *et al*. 2013). A thorough understanding of the mechanisms underlying these disorders would advance treatment innovation. As such, understanding the regulation of early, normal, decidual neoangiogenesis and vascular remodeling is essential.

The leukocytes represent a large proportion of cells in decidua basalis (DB), with uterine natural killer (uNK) cells accounting for 70% of the early decidual leukocytes (Bulmer *et al*. 1991, King & Loke 1991, Erlebacher 2013). uNK cells (also called decidual or dNK by many authors) were formerly called endometrial granulocytes in humans and several other species.

ISSN 1470–1626 (paper) 1741–7899 (online)

DOI: 10.1530/REP-14-0271

Online version via www.reproduction-online.org

In mice, they were known as granulated metrial gland or GMG cells. The considerable body of literature developed under the older names should not be ignored. uNK cells seem relatively analogous between mice and humans, except for the timing of their recruitment to the uterus. In each species, recruitment coincides with induction of decidualization, a pre-implantation event in humans but post-implantation in mice. Surface phenotypic markers are also distinctive. In humans, uNK cells are phenotypically $CD56^{bright}CD16^{-}$ in contrast to peripheral blood NK cells that are predominantly $CD56^{dim}CD16^{+}$. In mice, uNK cells can be separated into two subsets using the lectin *Dolichos biflorus* agglutinin (DBA). There is a unique decidual DBA^{+} subset that rapidly becomes the dominant population (Chen *et al*. 2012) and a splenic-like DBA^{-} subset (Yadi *et al*. 2008). In studies using adoptive transfer of normal mouse bone marrow to lymphocyte-deficient mice followed by mating, only DBA^{+} uNK cells was shown to differentiate. This indicates that the specialized, pregnancy-associated uNK cell subset differentiates from extra-uterine progenitor cells. The origin of DBA^{-} uNK cells has not been defined (Zhang *et al*. 2009, Chen *et al*. 2012, Felker *et al*. 2013). In both humans and mice, the functions of uNK cells differ from peripheral NK cells. Instead of predominant cytotoxic actions against virus-infected or cancerous cells, the uterine subsets show angiogenic and vessel remodeling activities. This review summarizes recent literature on uNK cell roles in decidual neoangiogenesis and SA remodeling. It also briefly discusses DB in mice lacking placental growth factor (PGF), a factor linking in humans with a of PE. There is significant information on the endocrine regulation of uNK cells (Muller *et al*. 1999, Henderson *et al*. 2003, Oh & Croy 2008, Cui *et al*. 2012, Li *et al*. 2013), which was not addressed in this review.

Role of NK cells in normal decidual neoangiogenesis

During early human and mouse pregnancies, decidual capillaries and arterioles develop by angiogenesis. Histological studies report that mouse uNK cells are placed spatially and temporally within the region of active microvascular development close to the organ-feeding uterine artery (Wang *et al*. 2000, 2003, Li *et al*. 2008, Degaki *et al*. 2012). Immunohistological studies revealed that uNK cells express numerous angiogenic factors. Human uNK cells express vascular endothelial growth factor A (VEGFA), VEGFC, PGF, angiopoietin 1 (ANGPT1), ANGPT2, matrix metalloproteinase 2 (MMP2), transforming growth factor beta 1 (TGFβ1), and NKG5 (currently known as granulysin) (Langer *et al*. 1999, Li *et al*. 2001, 2008, Hanna *et al*. 2006, Lash *et al*. 2011*a*). Murine uNK cells express VEGFA, VEGFC, PGF, delta-like ligand 1 (DLL1), TGFβ1, MMPs, tumor necrosis factor A, and inducible nitric oxide synthase (iNOS) (Chen *et al*. 1993, Hunt 1994, Burnett & Hunt 2000, Wang *et al*. 2000, 2003, Tayade *et al*. 2007, Naruse *et al*. 2009, Chen *et al*. 2012, Degaki *et al*. 2012).

Among the angiokines produced by uNK cells, the VEGF family is of central importance. It encodes seven distinct proteins (VEGFA, B, C, D, E, F, and PGF) with VEGFA as the main regulator of angiogenesis in numerous tissues. VEGFA is highly expressed in the uteroplacental unit, particularly during early pregnancy, and is an essential mediator of decidual angiogenesis (Kim *et al*. 2013). Indeed, VEGFA is the only family member characterized as embryonic lethal when genetically ablated in mice (Bellomo *et al*. 2000, Carmeliet *et al*. 2001, Karkkainen *et al*. 2004, Haiko *et al*. 2008). Furthermore, lethality ensued when only one allele was deficient (i.e. heterozygote; Ferrara *et al*. 1996). Three VEGF receptors are characterized: VEGFR1 (FLT1), VEGFR2 (KDR), and VEGFR3 (FLT4). VEGFA signals through VEGFR1 and VEGFR2. Independent knockout of each of the three VEGF receptors is lethal during mouse development (Shalaby *et al*. 1995, Fong *et al*. 1999, Haiko *et al*. 2008). However, mice lacking the tyrosine kinase domain but retaining the ligand-binding portion of VEGFR1 are viable (Hiratsuka *et al*. 1998). The uNK cells contribute greatly to early decidual expression of VEGFA, primarily from the $CD56^{bright}CD16^{-}$ subset in humans (Hanna *et al*. 2006) and the DBA^{+} subset in mice (Chen *et al*. 2012). It should be noted, however, that others have reported less production of VEGFA by uNK cells (Lash *et al*. 2006, Wallace *et al*. 2014). Like angiogenic processes in other tissues, the expression of VEGFA in uNK cells is induced by hypoxia (Cerdeira *et al*. 2013). Implantation site VEGFA is also contributed to by trophoblasts, uterine stromal cells, and endothelial cells; thus, the specific contributions of uNK cell-derived VEGFA to implantation sites are estimated indirectly and imprecisely from rodent NK cell depletion and reconstitution experiments.

PGF, another member of the VEGF family, is highly expressed in both human and mouse pregnancies (Torry *et al*. 1998, Tayade *et al*. 2007). Although PGF levels are highest during mid-pregnancy, and its deficiency in humans is linked with PE (Levine *et al*. 2004), genetic deletion of *Pgf* in mice is viable and fertile (Carmeliet *et al*. 2001). It was initially postulated that PGF functioned as an angiogenic factor by displacing VEGFA from the decoy receptor VEGFR1, allowing VEGFA to signal through VEGFR2 (Park *et al*. 1994). However, accumulating evidence suggests PGF participates in angiogenesis by numerous additional mechanisms. PGF upregulates the expression of angiogenic factors such as VEGFA, basic fibroblast growth factor, platelet-derived growth factor beta, and MMPs (Roy *et al*. 2005, Marcellini *et al*. 2006). PGF also stimulates mesenchymal fibroblast proliferation (Yonekura *et al*. 1999) and recruits myeloid progenitor cells (Hattori *et al*. 2002, Rafii *et al*. 2003)

and macrophages (Selvaraj *et al*. 2003) to the sites of neoangiogenesis.

Along with VEGF family signaling, NOTCH family signaling has multiple roles in both normal vascular development and pathological angiogenesis, including regulation of VEGFR1 (Jakobsson *et al*. 2009, Outtz *et al*. 2010, Krueger *et al*. 2011). NOTCH is involved in the differentiation of endothelial tip cells and vascular smooth muscle cells and regulates cell-fate decisions in arteriovenous differentiation (Gridley 2010). In mammals, the NOTCH receptor family has four members (NOTCH1–4) that bind five ligands encoded by delta-like (DLL1, DLL3, and DLL4), and Jagged (JAG1 and JAG2) gene families (Gridley 2010). NOTCH receptors and ligands are expressed throughout the placenta during pregnancy and play roles in fate determination of placental cell (De Falco *et al*. 2007). These proteins are downregulated in PE placentas (Cobellis *et al*. 2007). NOTCH1 and NOTCH2 are expressed on uNK cells, which secrete interferon gamma (IFNG) upon NOTCH activation (Manaster *et al*. 2010). NOTCH1 is essential for stromal decidualization in mice and its expression over pregnancy closely parallels the time course of uNK cell abundance (Afshar *et al*. 2012). In mice, only some DBA^+ uNK cells express DLL1. We postulate that the DBA^+DLL1^+ cells are at the center of the DB, where they serve as an exogenous DLL1 source for endothelial tip cell differentiation (Degaki *et al*. 2012). In humans, PGF is highly expressed by fetal trophoblasts, and also by $CD56^{bright}CD16^-$ uNK cells (Li *et al*. 2001, Lash *et al*. 2006), decidualized stromal cells (Ghosh *et al*. 2000), and endothelial cells (Hauser & Weich 1993). Similarly, mouse DBA^+ uNK cells express PGF (Chen *et al*. 2012) and it is reported essential for uNK cell cytokinesis (Tayade *et al*. 2007).

Angiogenic activities of uNK cells in early DB

In addition to angiokine expression by uNK cells, *in vivo* angiogenic activities of uNK cells are reported in mice. Our laboratory used the technique of whole-mount immunohistochemistry to stain vascular endothelium ($CD31^+$) in intact viable implantation sites from alymphoid $Rag2^{-/-}Il2rg^{-/-}$ mice (uNK/NK^-; T^-; B^-). The onset of angiogenesis in DB was delayed. Subsequently (GD8.5), impaired angiogenesis in the lateral vascular sinuses (venous drainage regions) was seen (Hofmann *et al*. 2014*a*). $Rag2^{-/-}Il2rg^{-/-}$ implantation sites were fully normalized by pre-conception transplantation with $Rag2^{-/-}$ (NK^+, T^-, B^-) bone marrow (Hofmann *et al*. 2014*a*). Using matings that tagged conceptus-derived cells with green fluorescent protein (GFP), it was apparent in the reconstituted mice, as well as in normal mice, that no interactions occurred between uNK cells and trophoblasts in the live tissues studied at these early times (Croy *et al*. 2012, Hofmann *et al*. 2014*a*,*b*).

Despite the early delays in implant site development, $Rag2^{-/-}Il2rg^{-/-}$ pregnancies are successful. From studies using ultrasound (Zhang *et al*. 2011) and chronic continuous radiotelemetry (Burke *et al*. 2010*a*) we postulate that mice achieve this through mid-to-late gestational cardiac adaptations of mothers and conceptuses. Of particular note, our $Rag2^{-/-}Il2rg^{-/-}$ whole-mount studies strongly implicated uNK cells in the process of pruning newly developed vascular plexuses into their mature shapes. The cytotoxic molecules synthesized by uNK cells are likely essential for pruning; however, to directly address this hypothesis, angiogenesis in implantation sites from mice deficient in such products, for example the perforin-null mouse (Stallmach *et al*. 1995), must be assessed. Insights gained from $Rag2^{-/-}Il2rg^{-/-}$ whole-mount studies caused us to re-interpret one of our widely cited earlier observations. At the time mice genetically depleted in NK cells were first reported, we described their decidua as edematous (Guimond *et al*. 1998). This is no longer our interpretation. Now, we interpret the very large anomalous spaces that become prominent across the DB from mid-gestation as larger than normal blood vessels that failed to develop appropriate levels of fine branching (Hofmann *et al*. 2014*a*).

Not only is frequency of uNK cells of importance in angiogenic processes but also activation status. NK cells are activated by a variety of ligands, ranging from viral proteins to major histocompatibility complex 1 (MHC1)-like and self MHC1 molecules. NK cells of natural cytotoxicity receptor 1 (*Ncr1*) gene disrupted mice ($Ncr1^{Gfp/Gfp}$) do not express a functional NCR1 (NKp46 in humans), and thus have poorly activated NK cells with reduced function. NCR1 is an activating receptor that ligates non-MHC-related molecules. Implantation sites in $Ncr1^{Gfp/Gfp}$ have normal uNK cell numbers, but whole-mount staining for CD31 shows less angiogenesis at GD6.5, absence of elevated protein expression around the embryonic crypt and delayed development of GFP-expressing conceptuses. Unexpectedly, although uNK cells are only present on the mesometrial side of the uterus, the GD8.5 anti-mesometrial vessels in $Ncr1^{Gfp/Gfp}$ mice were narrower than that in controls and their branching was disorganized (Felker *et al*. 2013). By GD8.5, $Ncr1^{Gfp/Gfp}$ DBA^+ uNK cells had greater immunoreactivity for VEGFA than controls (Felker, Lima and Croy, unpublished data).

Others studied GD8.5 implantation sites in mice treated with anti-NKG2D on GD6.5 and 7.5. NKG2D is an NK cell activation receptor that recognizes MHC class I-related molecules but not MHC itself (Raulet *et al*. 2013). Although flow cytometric studies report that NKG2D is more weakly expressed by angiogenic DBA^+ than by DBA^- uNK cells (Yadi *et al*. 2008), anti-NKG2D antibody treatment depleted DBA^+ uNK cells, decreased vessel density, and prevented vascular sinus formation in the central mesometrial decidua (Kim *et al*. 2013).

The processes promoting uterine lumen closure and anti-mesometrial angiogenesis were unaffected by NKG2D antibody depletion, but might have been affected if treatment had been started earlier in gestation or if the uNK cell depletion had been as absolute as achieved genetically.

The mouse LY49 receptor family contains NK cell activating and inhibiting receptors; LY49 receptors use classical MHC class I molecules as ligands. We assessed the overall contribution of LY49 receptor signaling to pregnancy in pan-knockdown LY49 mice (Lima *et al*. 2014). uNK cell numbers were not reduced, but knockdown of the gene family had a greater effect on the angiogenic DBA^+ uNK cell subset, reducing LY49 expression from 80% (controls) to 6% (GD9.5 pan-knockdown genotype). In contrast, LY49 expression by DBA^- uNK cells was reduced from 90% (controls) to 50% (pan-knockdown). Phenotypically, LY49-knockdown mice were infertile. This was characterized as frequent failure of well-developed blastocysts to implant. If pregnancy was established, LY49 knockdown resulted in lagging decidual angiogenesis and, in contrast to mice lacking NCR1, significantly reduced uNK cell production of VEGFA. Of interest, neither IFNG (intracytoplasmic FACS analysis) nor perforin immunohistochemistry (IHC analysis) was reduced in LY49 knockdown uNK cells (Lima *et al*. 2014). Thus, MHC recognition appears to be important for VEGF regulation, while recognition by other receptor pathways must be responsible for IFNG and perforin induction. Consistent between all of these mouse model studies is the importance of uNK cell function from the earliest stages of decidual angiogenesis.

In humans, *in vitro* assays using isolated, first trimester uNK cells substitute for early implantation site studies. These have elucidated angiogenic properties of early human decidual cells. One important study reported increased human umbilical vein endothelial cell (HUVEC) migration and tube formation in response to uNK cell supernatants (Hanna *et al*. 2006). Similarly, the vascularization of JEG3 choriocarcinoma tumors injected into mice was greater when uNK cells were co-transplanted (Hanna *et al*. 2006). VEGF and PGF were identified as important signalling molecules in these studies (Hanna *et al*. 2006). Using a carefully defined time course approach to study specimens from early elective terminations (Lash *et al*. 2006), Lash *et al*. reported that human uNK cells produced higher levels of the angiogenic factors VEGFC and ANGPT1 at 8–10 weeks gestation than at 12–14 weeks; the levels of PGF and TGFβ1 were low and did not differ significantly between these two times. A more recent study has suggested that the angiogenic functions of uNK cells are regulated by sphingosine-1-phosphate (S1P), a circulating bioactive lipid which modulates vascular tone and immune cell behaviour (Cyster & Schwab 2012, Kerage *et al*. 2014). uNK cells express S1P receptor 5 and respond to S1P signaling by altering their angiogenic functions (Zhang *et al*. 2013). HUVEC tube formation in response to uNK cells or uNK cell-conditioned media was decreased if the uNK cells were pre-treated with an inhibitor of S1P signalling (Zhang *et al*. 2013). Although Zhang *et al*. (2013) suggest that a single mechanism regulates angiogenic functions of uNK cells, more research is needed to address this question. Kim *et al*. (2013) conclude from studies using a VEGF-trap approach that hypoxia is not a regulator of angiogenesis in early mouse decidua.

Roles of NK cells in physiological changes to maternal SA

Once the interval of decidual angiogeneis and embryonic development is complete, pregnancies enter the gestational interval of rapid fetal growth. This phase places even greater demands upon the maternal cardiovascular system. In both humans and mice, transition to the growth phase coincides with opening of the placental circulation. This is achieved through mechanisms that include SA remodeling, the terminal branches of the major maternal uterine artery (Leonard *et al*. 2013). In humans, a large numbers of SA supply the intervillous space; most but not all are typically remodeled. Scoring of remodeling in placental bed biopsies depends upon gestational time, relative position along the length of the vessel as well as position relative to the placental midline with the vessels most distal to the conceptus the last to be remodeled (Brosens *et al*. 2002). SA remodeling normally occurs between weeks 7 and 18 in human pregnancy (Lash *et al*. 2006, Pijnenborg *et al*. 2006). In mice, 5–10 SA converge at the layer of the trophoblast giant cells to form a small number of central arterial canals leading to the exchange area of the placental labyrinth (Adamson *et al*. 2002). SA remodeling accompanies opening of the placental circulation at ~GD9.5 in mice (Adamson *et al*. 2002, Burke *et al*. 2010*b*, Croy *et al*. 2012, Leonard *et al*. 2013).

While fetal extravillous trophoblasts (EVTs) contribute to SA remodeling in both humans and mice, earlier preparation of the vessels is mediated by immune cells, especially uNK cells. It is now held that both maternally-derived and conceptus-derived mechanisms and physical properties, such as mechano-sensing by endothelial cells (James *et al*. 2012), contribute to the apoptosis in vascular smooth muscle cells and endothelial cells lining these high-resistance vessels (Ashton *et al*. 2005, Wallace *et al*. 2012). This phase is becoming known as 'trophoblast-independent remodeling' (Smith *et al*. 2009, Robson *et al*. 2012, Wallace *et al*. 2012). In humans, loss of vascular smooth muscle cells is accompanied by the influx of EVTs to envelope the arteries. These vessel-associated EVTs deposit extracellular matrix called fibrinoid that stabilizes the dilated,

venous-like vessels (Smith *et al*. 2009, Hazan *et al*. 2010, Croy *et al*. 2011, Cerdeira & Karumanchi 2012, Robson *et al*. 2012, Wallace *et al*. 2012). The dogma currently held is that modified SAs are no longer under maternal vasomotor control. Our intravital microscopic studies comparing SA responses in mice with vasoactive compounds before (GD8) and after (GD12) modification challenge this idea because vasoconstrictive responses were unaltered by modification despite much larger arterial lumen diameters and the absence of detectable (by IHC) vascular smooth muscle (Leonard *et al*. 2013). Interestingly, intravital microscopy studies on mouse brain vessels in an Alzheimer's model implicate monocytes in the deposition of extracellular matrix around arteries (Michaud *et al*. 2013). With simultaneous use of three different fluorescent gene tags, these authors observed normal continuous clearance of vascular debris by monocytes in veins. In pathology, this mechanism is overwhelmed and extracellular material is deposited in excess around arteries. Venous monocyte clearance might be a physiological mechanism in decidua with the gain in SA amyloid as a parallel, oversupply process. Amyloid deposition may be a mechanism for strengthening dilated vessels to accommodate the increased systemic maternal cardiovascular output needed to support mid pregnancy (Hunter & Robson 1992, Collins *et al*. 2012). This amyloid deposition process may occur in a transient fashion, making detection by traditional histological techniques difficult. The continued adaptation and use of live imaging systems to address questions surrounding vascular, immune cell, and trophoblast interactions in mice and in human cell cultures hold great promise for refining, modifying, and improving current understanding of the maternal–fetal interface (Schmerse *et al*. 2014).

Our studies in multiple strains of NK cell-deficient mice without and following NK cell lineage reconstitution were the first to identify uNK cells as the agents of trophoblast-independent SA remodeling (Croy *et al*. 2011). Although many factors secreted by uNK cells around the time of remodeling have been implicated as possible triggers of these changes in vascular structure, we focused on the role of uNK cell-produced IFNG (Ashkar *et al*. 2000). Results from alymphoid mice treated with mrIFNG and confirmed in mice with uNK cells lacking the genetic ability to produce IFNG indicated that IFNG alone, independent of the presence of uNK cells, is sufficient to induce SA remodeling (Ashkar *et al*. 2000, Ashkar & Croy 2001). IFNG synthesis by uNK cells is induced *in vivo* by interleukin 12 (IL12) and enhanced by IL18 (Zhang *et al*. 2003, Murphy *et al*. 2009). In mouse mesometrial decidua, IFNG increases from negligible, nonpregnant values to detectable levels at GD6.5. The levels increase four- to sixfold to a peak at GD10.5, then drop at GD12.5–14.5, the period after SA remodeling (Ashkar *et al*. 2000, Zhang *et al*. 2003, Murphy *et al*. 2009). Although rising IFNG levels correspond with increasing total uNK cell numbers, the DBA^- subset that is proportionally diminished during this interval is the primary IFNG source (Chen *et al*. 2012).

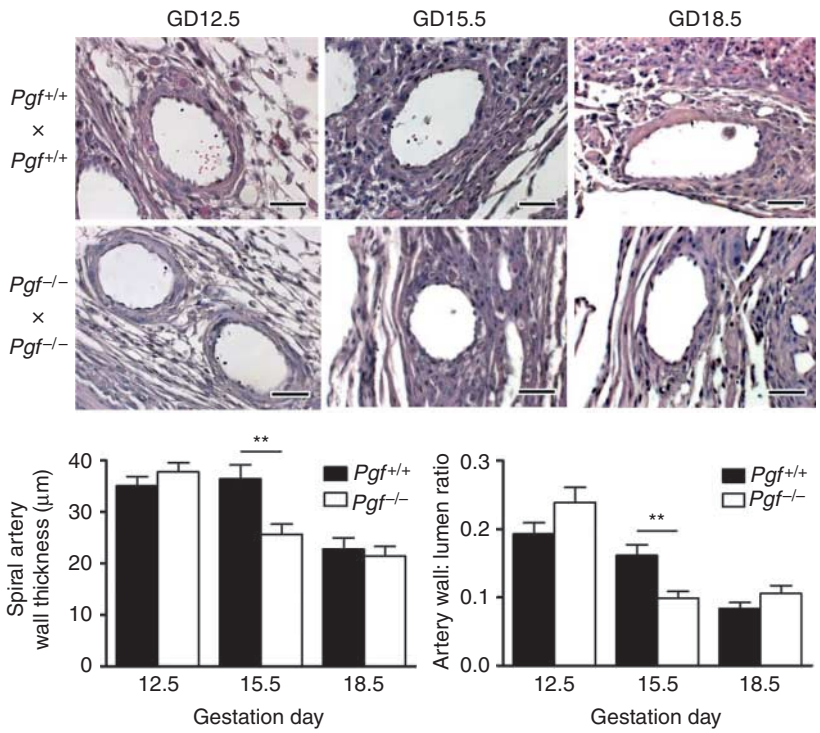

Figure 1 Decidual SA remodeling is delayed in $Pgf^{-/-} \times Pgf^{-/-}$ compared with $Pgf^{+/+} \times Pgf^{+/+}$ implantation sites. $Pgf^{+/+} \times Pgf^{+/+}$ and $Pgf^{-/-} \times Pgf^{-/-}$ mice at GD12.5, 15.5, and 18.5 were anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Placentas were harvested, processed for paraffin embedding, sectioned at 6 μm, and stained with hematoxylin and eosin. Photomicrographs in the DB region were taken and SA wall thicknesses and lumen diameters were quantified. Upper panels display representative sections of DB from mice of each genotype and timepoint. Lower panels display quantification of SA wall thickness (left) and wall: lumen ratio (right). Three implantation sites from each of three pregnancies/genotype/GD were studied. For each implantation site, three to five sections were measured. Photomicrographs were captured using a Zeiss epifluorescence microscope with Axiovision SE64 Rel 4.8 (Carl Zeiss, Oberkochen, Germany) and analyzed using ImageJ Software (NIH, Bethesda, MD, USA). $^{**}P<0.01$ vs $Pgf^{+/+}$ genotype at the corresponding timepoint. Scale bars represent 100 μm.

IFNG is responsible for regulating the expression of >0.5% of the mouse genome, including genes important for vascular smooth muscle cell proliferation, cell adhesion, regulation of vascular contractility, and cellular apoptosis (Ashkar & Croy 2001, Murphy *et al.* 2009). We postulate that IFNG acts indirectly by altering gene expression differentially within the cell types that comprise and support vessels. IFNG-regulated VEGF, iNOS, and alpha 2-macroglobulins, a family of IFNG-regulated protease inhibitors, are among the most differentially upregulated genes at mid-gestation in mice (He *et al.* 2005). Alpha 2-macroglobulins limit the rate of EVT invasion and bind molecules, including VEGF, that affect SA dilation and elongation (Ashkar & Croy 2001, Croy *et al.* 2003, Esadeg *et al.* 2003).

In human uNK cells, ANGPT1 and ANGPT2 are expressed to a high degree and regulate normal SA remodeling and placentation (Li *et al.* 2001, Lash *et al.* 2006). Isolated uNK cells express more ANGPT1 and ANGPT2 at 8–10 weeks of gestation compared with 12–14 weeks; however, ANGPT2 is expressed significantly more than ANGPT1 (Lash *et al.* 2006). *In vitro* models, placental angiogenesis suggest that ANGPT1 and ANGPT2, along with IFNG and VEGFC, disrupt vascular smooth muscle cell integrity to contribute to early angiogenesis and SA remodeling (Robson *et al.* 2012). In other tissues and models of angiogenesis, ANGPT1 and ANGPT2 act by stabilizing endothelial cell tight junctions, and may counteract vascular leakage induced by VEGFA (Suri *et al.* 1996, Fukuhara *et al.* 2008, Koh 2013). This function of ANGPT1 and ANGPT2 has yet to be validated in mouse or human uteroplacental angiogenesis.

uNK cells and pregnancy complications

Human

Direct links have been suggested between improper uNK cell-promoted decidual angiogenesis and human reproductive health. High uNK cell numbers (>5%) in secretory phase endometrial biopsies are linked with an increase in decidual vessel density in women suffering from RSA (Quenby *et al.* 2009). Excessive decidual angiogenesis in early pregnancy is postulated to lead to increased oxidative stress in the conceptus as a mechanism underlying RSA. Rather than number of vessels, it may be vessel maturity and differentiation that are important for blood flow. In both normal women and those with RSA, uNK cell numbers are inversely correlated with the number of vessels surrounded by mature myosin-expressing vascular smooth muscle cells. These vessels lead to high-resistance indices upon ultrasound examination (Quenby *et al.* 2009). Clinical trials were initiated to examine prednisolone as an intervention to decrease uNK cell number and consequently RSA (Lash *et al.* 2011*b*). Prednisolone decreased uNK cell number in certain women and, in treated women who subsequently had a successful pregnancy, secretory endometrial vessel density was decreased (Lash *et al.* 2011*b*).

In another report on human pregnancy termination specimens (mean 7 weeks gestation), decidua from patients electing termination was compared with that from terminations for fetal demise (missed abortions). In the latter, lower vessel density was found in decidua

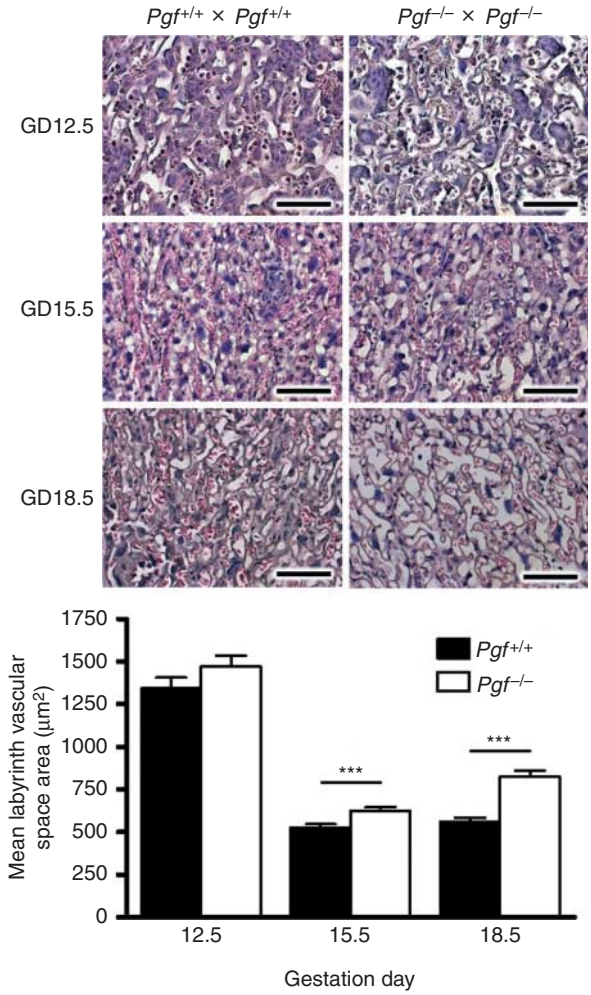

Figure 2 $Pgf^{-/-} \times Pgf^{-/-}$ placentas are deficient in labyrinthine vascular branching. $Pgf^{+/+} \times Pgf^{+/+}$ and $Pgf^{-/-} \times Pgf^{-/-}$ mice at GD12.5, 15.5, and 18.5 were anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde. Placentas were harvested, processed for paraffin embedding, sectioned at 6 μm, and stained with hematoxylin and eosin. Photomicrographs were taken and vascular spaces in the labyrinth region quantified. Top panels display representative sections of the labyrinth region from mice of each genotype and timepoint. Bottom panels display quantification of the vascular space area. Three implantation sites from each of three pregnancies/genotype/GD were studied. For each implantation site, three to five sections were measured. Photomicrographs were captured using a Zeiss epifluorescence microscope with Axiovision SE64 Rel 4.8 (Carl Zeiss) and analyzed using ImageJ Software (NIH). ***$P<0.001$ vs $Pgf^{+/+}$ genotype at the corresponding timepoint. Scale bars represent 50 μm.

parietalis and DB, although VEGFA and ANGPT2 expression were higher in DB (Plaisier *et al.* 2009). $CD56^{bright}CD16^{-}$ uNK cells were increased in aspirated, decidua-associated endometrium that morphologically resembled secretory-phase endometrium of the missed abortion patients, but not decidua parietalis or DB (Plaisier *et al.* 2009). The interpretation of the results from this study highlight the difficulties of addressing cause, effect, or compensatory responses of uNK cells in patients and the value of animal models in studies of early implantation site angiogenesis.

Remodeling of SA has been deemed essential for healthy human pregnancy, as deficits in this process are linked with pregnancy complications including PE, FGR, and preterm labor (Robson *et al.* 2012). In a clinical study examining placental beds obtained during elective caesarean section, major defects, absent in normal pregnancy, were seen in SA in samples from PE and FGR pregnancies (Lyall *et al.* 2013). Mechanistically, IFNG levels are elevated in the plasma, peripheral leukocytes, and decidua of women with pregnancies complicated by PE (Murphy *et al.* 2009). Although IFNG is important in SA remodeling, overabundance may impair EVT invasion and disturb normal angiogenic processes. Our studies in NK/uNK cell deficient mice give results inconsistent with currently accepted ideas concerning SA remodeling (Croy *et al.* 2011). Our data suggest that additional mechanisms must be superimposed upon the nonremodeled SA phenotype to result in hypertension or other major adverse gestational outcomes. Through the use of continuous radiotelemetry or daily ultrasound studies to monitor mouse cardiovascular systems, we now hypothesize that the key outcome from SA remodeling is cardiac protection of the mother. Furthermore, when SA do not modify, the placental and fetal cardiovascular systems become compromised, resulting in conceptus compensations and adaptions that persist to term and likely postnatally as fetal programming effects (Burke *et al.* 2010*b*).

Mice: PGF deficiency

Low plasma PGF in early to mid-pregnancy was recently postulated to be the central marker for distinguishing between two distinct pathogenic processes leading to clinical PE presentation (Powers *et al.* 2012, Staff *et al.* 2013). Although deficiency in PGF during pregnancy is implicated in the more severe PE phenotype, the mechanisms by which low PGF contributes to these effects remain unclear (Levine *et al.* 2005, Verlohren *et al.* 2010). Also unclear are potential roles for low gestational PGF in the elevated *postpartum* cardiovascular risks seen in women and children who experienced PE pregnancies (Davis *et al.* 2012, Ray *et al.* 2012, McDonald *et al.* 2013, Tuovinen *et al.* 2013). Our studies of pregnancies in $Pgf^{-/-}$ mice found that PGF regulates uNK cell cytokinesis (Tayade *et al.* 2007). Ultrastructural analyses of GD8.5 $Pgf^{-/-}$ uNK cells additionally identified aberrant features such as irregularly shaped granules and looping endoplasmic reticulum (Rätsep *et al.* 2014). Importantly, the ultrastructural appearance of $Pgf^{-/-}$ uNK cells differs from that of mature, senescent secretory NK and uNK cells which promote angiogenesis (Paffaro *et al.* 2003, Rajagopalan & Long 2012). Deficient vascular branching is present at GD6.5–9.5 in $Pgf^{-/-} \times Pgf^{-/-}$ decidua (Rätsep *et al.* 2014) and SA remodeling is delayed until GD14 (Fig. 1). Less vascular branching is present in the $Pgf^{-/-} \times Pgf^{-/-}$ placental labyrinth at GD15.5–18.5, which would limit surface area for maternal–fetal nutrient and waste exchanges (Fig. 2).

Efforts to measure gestational blood pressures in $Pgf^{-/-}$ mice by radiotelemetry were unsuccessful due to CNS pathologies that developed after the carotid arterial surgery required for radiotransmitter placement. Resin casting of the $Pgf^{-/-}$ brain arterial system revealed that PGF has a major role in the optimization of fetal brain angiogenesis. $Pgf^{-/-}$ brain arteries were highly disorganized and abnormally patterned. Furthermore, 80% of animals had an incomplete circle of Willis (Fig. 3) that

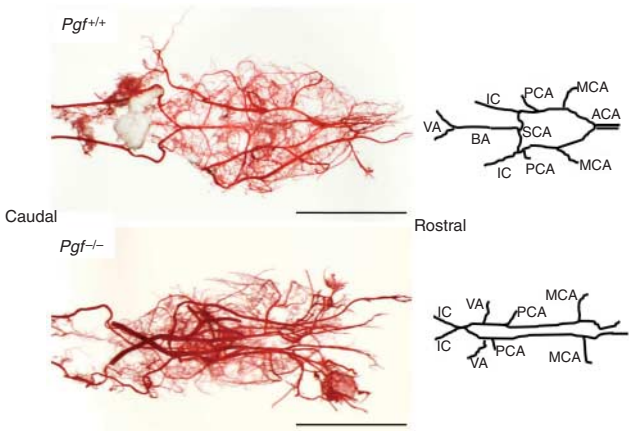

Figure 3 Resin casts of the cerebral arterial system. Nonpregnant adult $Pgf^{+/+}$ and $Pgf^{-/-}$ females were anesthetized with sodium pentobarbital and transcardially perfused using 140 mM NaCl, 10 mM KCl, and 5 mM EDTA solution (pH 7.5) to remove intravascular blood. Mice were subsequently injected through the thoracic aorta with 2 ml Batson's #17 polymer (Polysciences, Inc., Warrington, PA, USA) which was allowed to polymerize for 24 h. The surrounding tissue was subsequently digested away in 1 M NaOH and 5% Contrad 70 detergent (Fisher Scientific, Pittsburgh, PA, USA) for ~8 weeks. The resulting vascular casts (seven per genotype) were photographed using a Zeiss dissecting microscope (Carl Zeiss). $Pgf^{-/-}$ brain vasculature was disorganized and deficient in fine branching compared with controls. Commonly (>80%) $Pgf^{-/-}$ brain vasculature had incomplete Circle of Willis (compare between the line drawings representing the major vessels in each cast to the right). The $Pgf^{-/-}$ vascular field is narrower and more elongated than $Pgf^{+/+}$, suggesting alterations in normal brain anatomy and in skull shape. $Pgf^{-/-}$ casts show an unusual central prominence of large vessels. Scale bars represent 1 cm. ACA, anterior cerebral artery; BA, basilar artery; IC, internal carotid; MCA, middle cerebral artery, PCA, posterior cerebral artery; SCA, superior cerebellar artery; VA, vertebral artery.

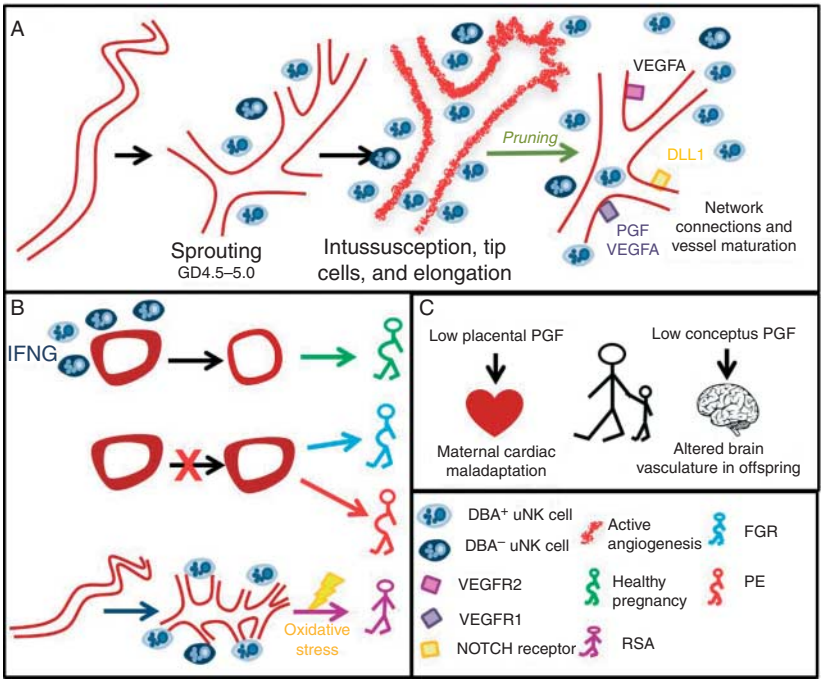

Figure 4 Schematic diagram of angiogenic processes driven by uNK cells during healthy and complicated pregnancies. In normal pregnancies (A), DBA^+ uNK cells produce angiogenic factors such as VEGFA, PGF, and DLL1 and enhance vessel sprouting at GD4.5–5.0. This phase is followed by intussusception, tip cell differentiation, and elongation that are all scheduled 'on time' in the presence of uNK cells and delayed in uNK cell deficiency. Later, pruning and orthogonal branching occurs through endothelial VEGFR1, VEGFR2, and NOTCH receptors leading to vascular network connections and vessel maturation. Branching is deficient in DB of mice lacking uNK cells. (B) In healthy pregnancy, IFNG produced by DBA^- uNK cells induces SA remodeling. In the absence of SA remodeling, FGR and/or PE are common. Conversely, increased uNK cell numbers and excessive angiogenesis causing oxidative stress are linked with RSA. (C) Low placental production of PGF during late pregnancy limits maternal cardiovascular adaptation. Similarly, low conceptus production of PGF alters vascular structures in some inner cell mass-derived fetal organs including the brain. DBA, *Dolichos biflorus* agglutinin; DLL1, delta-like ligand 1; FGR, fetal growth restriction; IFNG, interferon gamma; IL, interleukin; PE, preeclampsia; PGF, placental growth factor; RSA, recurrent spontaneous abortion; SA, spiral artery; VEGF, vascular endothelial growth factor.

combined with additional anomalies of the internal carotid artery (MT Rätsep, N Peterson, AY Jin & BA Croy, unpublished data), account for our poor surgical outcomes. These data may aid in explaining the increased tendency to suffer strokes, the reduction in cognitive ability, and vulnerability to depression reported in children born from a preeclamptic gestation (Hakim *et al*. 2013). Thus, although PGF expression is not necessary to initiate placental and fetal angiogenesis, its deficiency clearly results in sub-optimal vascular development of great importance during pregnancy.

Conclusions

Intricate linkages exist between early decidual angiogenesis, mid-pregnancy SA remodeling, and normalcy of pregnancy outcome. We argue that uNK cells are pivotal players in normal decidual angiogenesis and SA remodeling as summarized in Fig. 4. They act as supervisors for building the early decidual vasculature, ensuring the spatial and temporal coordination of many cell types and products to produce a well-supported placenta. While others using mouse decidual microarray analyses have reached a different conclusion (Bany *et al*. 2012), we find the morphological and genetic data reviewed here to be convincing. Our recent work (Hofmann *et al*. 2014*a*) and previous histological studies (Greenwood *et al*. 2000, Ashkar *et al*. 2003, Degaki *et al*. 2012, Lima *et al*. 2012) have highlighted the role of uNK cells in regulating the optimal timing and progression of decidual angiogenesis, a process that would not be detected through microarray analysis. Gaps still remain in our knowledge of the angiogenic processes occurring at the maternal–fetal interface and how these local processes are integrated into the systemic physiological changes to the pregnant female's cardiovascular system. With such knowledge, much of which can be gained from *in vivo* studies of mouse models using newer live tissue and intravital approaches, we will advance toward greater understanding of and hopefully improved clinical management for pregnancy disorders such as PE.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

Funding

M T Rätsep is supported by a Frederick Banting and Charles Best Canada Graduate Scholarship. The studies were supported by Awards from NSERC, CIHR, the Canada Research Chair's program and CFI (B A Croy).

Acknowledgements

The authors thank Dr Patricia Lima (Ottawa), Dr Scott Gerber (Rochester), Rayana L Luna (Queen's), and Nicole Ventura (Queen's) for helpful discussions.

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Received 27 May 2014
First decision 15 July 2014
Revised manuscript received 10 October 2014
Accepted 22 October 2014