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The role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding

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BACKGROUND: Human pregnancy requires robust hemostasis to prevent hemorrhage during extravillous trophoblast (EVT) invasion of the decidualized endometrium, modification of spiral arteries and post-partum processes. However, decidual hemorrhage (abruption) can occur throughout pregnancy from poorly transformed spiral arteries, causing fetal death or spontaneous preterm birth (PTB), or it can promote the aberrant placentation observed in intrauterine growth restriction (IUGR) and pre-eclampsia; all leading causes of perinatal or maternal morbidity

and mortality. In non-fertile cycles, the decidua undergoes controlled menstrual bleeding. Abnormal uterine bleeding (AUB) accompanying progestin-only, long-acting, reversible contraception (pLARC) accounts for most discontinuations of these safe and highly effective agents, thereby contributing to unwanted pregnancies and abortion. The aim of this study was to investigate the role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding.

METHODS: We conducted a critical review of the literature arising from PubMed searches up to December 2015, regarding *in situ* and *in vitro* expression and regulation of several specific proteins involved in uterine hemostasis in decidua and cycling endometrium. In addition, we discussed clinical and molecular mechanisms associated with pLARC-induced AUB and pregnancy complications with abruptions, chorioamnionitis or pre-eclampsia.

RESULTS: Progestin-induced decidualization of estradiol-primed human endometrial stromal cells (HESCs) increases *in vivo* and *in vitro* expression of tissue factor (TF) and type-1 plasminogen activator inhibitor (PAI-1) while inhibiting plasminogen activators (PAs), matrix metalloproteinases (MMPs), and the vasoconstrictor, endothelin-1 (ET-1). These changes in decidual cell-derived regulators of hemostasis, fibrinolysis, extracellular matrix (ECM) turnover, and vascular tone prevent hemorrhage during EVT invasion and vascular remodeling. In non-fertile cycles, progesterone withdrawal reduces TF and PAI-1 while increasing PA, MMPs and ET-1, causing menstrual-associated bleeding, fibrinolysis, ECM degradation and ischemia. First trimester decidual hemorrhage elicits later adverse outcomes including pregnancy loss, pre-eclampsia, abruption, IUGR and PTB. Decidual hemorrhage generates excess thrombin that binds to decidual cell-expressed protease-activated receptors (PARs) to induce chemokines promoting shallow placentation; such bleeding later in pregnancy generates thrombin to down-regulate decidual cell progesterone receptors and up-regulate cytokines and MMPs linked to PTB. Endometria of pLARC users display ischemia-induced excess vasculogenesis and progestin inhibition of spiral artery vascular smooth muscle cell proliferation and migration leading to dilated fragile vessels prone to bleeding. Moreover, aberrant TF-derived thrombin signaling also contributes to the pathogenesis of endometriosis via induction of angiogenesis, inflammation and cell survival.

CONCLUSION: Perivascular decidualized HESCs promote endometrial hemostasis during placentation yet facilitate menstruation through progestational regulation of hemostatic, proteolytic, and vasoactive proteins. Pathological endometrial hemorrhage elicits excess local thrombin generation, which contributes to pLARC associated AUB, endometriosis and adverse pregnancy outcomes through several biochemical mechanisms.

Key words: decidual cells / tissue factor / thrombin / contraception / uterine bleeding / pre-eclampsia / preterm birth

Introduction

Human reproduction is associated with significant risks of hemorrhage during blastocyst implantation and development of the primitive uteroplacental lacunar circulation as well as subsequent co-option of the maternal blood supply via remodeling of spiral arteries and arterioles by endovascular trophoblasts and decidual natural killer (dNK) cells (Hanna *et al.*, 2003; Macklon and Brosens, 2014). The latter immune system initiate the early steps of vascular remodeling by elaborating interferon-γ and angiogenic factors including vascular endothelial cell growth factor (VEGF)-C, the angiopoietins (Ang-1 and Ang-2) (Robson *et al.*, 2012; Sharkey *et al.*, 2015) and matrix metalloproteinases (MMPs), (MMP-2 and MMP-9) (Naruse *et al.*, 2009). An even greater hemostatic burden is posed by parturition, when, following delivery, placental detachment and expulsion occurs. Conversely, the absence of pregnancy leads to the controlled bleeding of menstruation. Over the past 25 years, our laboratory has sought to increase understanding of these paradoxical physiologic properties of human endometrium by focusing on the elaboration of hemostatic, angiogenic and immuno-regulatory cytokines as well as proteases and their inhibitors by decidualized endometrial stromal cells.

In contrast with the physiological occurrence of menstruation, pathological bleeding into the decidualized endometrium of pregnancy triggers abruption [aka decidual hemorrhage]-associated preterm birth (PTB). Moreover, derangements in the expression of decidual cell-derived modulators of hemostasis, angiogenesis and immune response are implicated in the shallow placentation associated with abruptions as well as other adverse pregnancy outcomes including intrauterine growth restriction (IUGR), pre-eclampsia and idiopathic spontaneous PTB. Taken together these abnormal conditions are strongly associated with maternal and/or perinatal morbidity and mortality as well as enormous health care costs and are linked to accelerated rates of cardiovascular mortality later in life among affected mothers (Lykke *et al.*, 2010b).

Progestin-only long-acting reversible contraceptives (pLARCs) are safe, highly effective and invaluable for use in women with preexistent thrombophilias in whom estrogen-containing agents are contraindicated. Administration of pLARCs is strongly associated with reduced rates of teenage pregnancy and abortion (Connolly *et al.*, 2014; Secura *et al.*, 2014). Moreover, pLARCs are relatively inexpensive making them ideal for use in less developed countries. However, pLARC-associated abnormal uterine bleeding (AUB) is generally not a health issue, but rather a source of personal inconvenience and annoyance and, in some societies, of religious taboo. It is the primary reason for discontinuation of these otherwise safe and effective agents (Short *et al.*, 2014). Such AUB is linked to derangements in the physiological regulation of mediators of endometrial hemostasis, angiogenesis, vasoconstriction and inflammation.

Methods

This manuscript is a critical review of the literature arising from PubMed searches up to December 2015, regarding *in situ* and *in vitro* expression and regulation of several specific proteins involved in uterine hemostasis in decidua and cycling endometrium. In addition, we discussed clinical and molecular mechanisms associated with pLARC-induced AUB and pregnancy complications with abruptions, chorioamnionitis or pre-eclampsia.

Menstrual cycle physiology

Tissue factor expressed by decidualized endometrial stromal cells mediates uterine hemostasis

During a woman's reproductive years, ovarian derived circulating estradiol and progesterone induce cyclical changes in the endometrium in preparation for blastocyst implantation (Jabbour *et al.*, 2006; Henriet *et al.*, 2012). During the proliferative (follicular) phase of the menstrual cycle, rising circulating estradiol levels prime the endometrium for the actions of progesterone whose circulating levels are increased following ovulation and maintained at elevated levels during the secretory (luteal) phase to induce decidualisation of human endometrial stromal cells (HESCs). The decidualisation process is accompanied by marked changes in cellular differentiation; i.e. conversion of fibroblast-like HESCs into epithelial-like decidual cells surrounded by an extracellular matrix (ECM) enriched in basement-membrane-like proteins such as collagen IV and laminin (Wewer *et al.*, 1985; Church *et al.*, 1996; Iwahashi *et al.*, 1996; Schatz *et al.*, 1999). During decidualisation, more than 33 epigenetic effector genes are modulated in HESCs suggesting that complex epigenetic modification provide a wide range of plasticity required for transformation of HESCs to decidual cells (Grimaldi *et al.*, 2012). Moreover, impaired decidualisation is strongly associated with recurrent pregnancy loss, suggesting the importance of proper decidual cell maturation in maintaining pregnancy (Salker *et al.*, 2010; Lucas *et al.*, 2015). Accompanying decidualisation, several key hemostatic proteins exhibit an increased expression in HESCs (Christian *et al.*, 2001). Chief among these is tissue factor (TF), the primary initiator of coagulation (Lockwood *et al.*, 1993a). TF is a 47 kDa trans-cell membrane glycoprotein member of the class-2 cytokine receptor family comprised of a hydrophilic extracellular domain, a membrane-spanning hydrophobic domain and a cytoplasmic tail. Under physiological conditions, endothelial cells do not express TF, whereas constitutive expression of TF in the adventitia of arteries and veins and in organ parenchymal cells forms a protective hemostatic envelope (Drake *et al.*, 1989; Lockwood *et al.*, 1993a; Mackman, 2004). As depicted in the coagulation cascade in Fig. 1, disruption of vascular integrity results in binding of cell membrane-bound TF to circulating plasma-derived factor VII or its active form, FVIIa. Consequently, factor Xa is generated directly by TF/factor VIIa, or indirectly via TF/VIIa activation of factor IX to IXa, which binds to its co-factor, factor VIIIa, to generate factor Xa. By either route, newly generated factor Xa binds to its co-factor factor Va to cleave prothrombin to thrombin (Bach, 1988). Once formed, thrombin converts fibrinogen to fibrin and activates

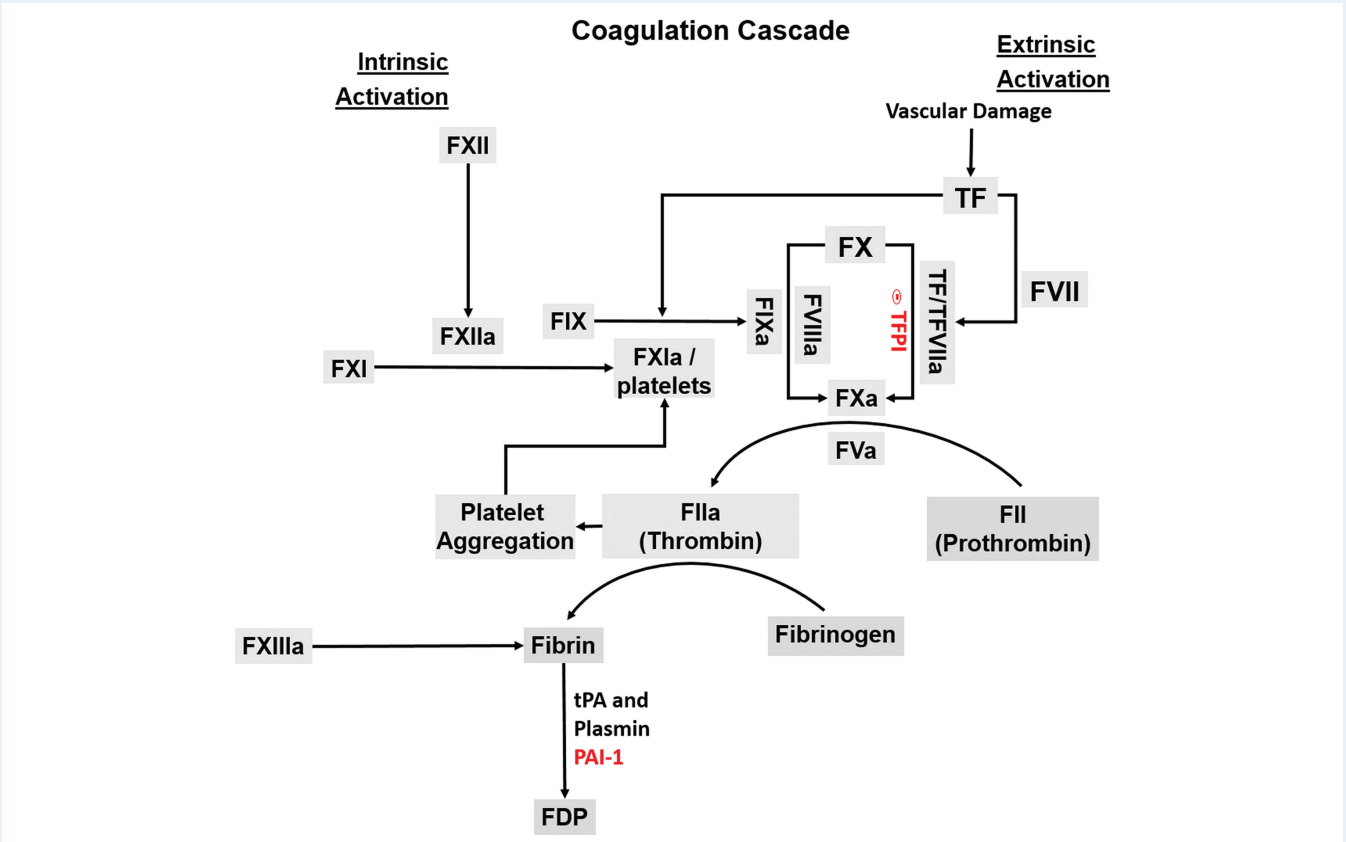

Figure 1 Schematic demonstration of the coagulation cascade. The tissue factor (TF)–factor (F)VIIa complex of the extrinsic pathway initiates blood coagulation by activating factor (F) X directly or indirectly by activating FIX of the intrinsic pathway. Thrombin plays a pivotal role in this process by activating several proteases and cofactors. Specifically, thrombin cleaves fibrinogen to soluble fibrin monomers. These are cross-linked by FXIIa and thrombin activated platelets (not shown) to form the fibrin clot. Fibrin is degraded into the fibrin degradation products (FDP) by plasmin formed by tissue type plasminogen activator (tPA). The action of tPA is regulated by its fast inactivator, plasminogen activator inhibitor type (PAI)-1.

platelets to promote hemostasis. The fibrin clot is then subjected to degradation by tissue type plasminogen activator (tPA) to restore vascular fluidity (Draxler and Medcalf, 2015). Premature fibrinolysis is prevented by plasminogen activator inhibitor type-1 (PAI-1), giving it a key role to play in modulating hemostasis (Sobel and Schneider, 2004).

Thrombin also stimulates cells by binding to transmembrane protease-activated receptors (PARs). The PAR family is comprised of four members, PAR1, PAR2, PAR3 and PAR4. The expression of PARs on the surface of various cell types mediates several cellular effects including angiogenesis, cell proliferation and apoptosis, cytokine release and inflammation (Fu *et al.*, 2015). For example, thrombin activation of PAR4 is implicated in promoting inflammation by affecting neutrophil recruitment, edema and plasma extravasation (Fu *et al.*, 2015). In endometrial stromal cells, PAR1 activation increases expression of VEGF, MMPs, both tPA and urokinase plasminogen activator (uPA) as well as interleukin (IL)-8 and macrophage/monocyte chemoattractant protein (MCP)-1 to exert respective effects on angiogenesis and ECM degradation as well as the endometrial neutrophil and macrophage population (Osuga *et al.* 2012a).

Compensating for the absence of a canonical progesterone response element in the TF gene promoter, the induction of TF by progesterone in HESCs occurs via enhanced expression of the transcription factor, Sp1 (Krikun *et al.*, 2000b) and requires the presence of epidermal growth factor (EGF) whose receptor is enhanced by progesterone in HESCs (Lockwood *et al.*, 2000). The pivotal role played by HESC-expressed TF in reproductive hemostasis is underscored by observations that: (i) no human genetic TF deficiency has been described; and (ii) knocking out the TF gene results in abnormal yolk sac-derived circulation, hemorrhage and embryonic lethality by day 8.5 in mice (Carmeliet *et al.*, 1996). Conversely, incorporation of a human TF mini-gene expressing 1% of wild type TF levels proved sufficient to rescue such TF knockout mice; although these low TF expressing mice when pregnant experience formation of placental labyrinth blood pools and high rates of lethal post-partum hemorrhage (Parry *et al.*, 1998). Finally, progesterone stimulation of TF expression in HESCs continues throughout pregnancy protecting against bleeding; compared with the evanescent induction of the TF gene in all other reported tissues and cell types, this phenomenon represents a biologically unique effect (Lockwood *et al.*, 2009). This sustained HESC expression of TF likely contributes to peripartum hemostasis.

Other factors produced by decidualized HESCs also promote endometrial hemostasis

Accompanying increased TF expression, decidualisation is also associated with enhanced HESC production of PAI-1, which acts as the primary anti-fibrinolytic agent by inhibiting the action of tPA, and also serves as an inhibitor of the ECM-degrading uPA (Schatz and Lockwood, 1993). Consistent with regulation of TF expression, the induction of PAI-1 gene transcription by progesterone is mediated by the Sp1 transcription factor and requires the presence of both EGF and its receptor (EGFR) (Lockwood *et al.*, 2000; Lockwood, 2001; Krikun and Lockwood, 2002). Concomitantly, progesterone promotes stabilization of the endometrial vasculature by down-regulating HESC-derived MMPs, a family of zinc-requiring enzymes that includes collagenases, gelatinases and stromelysins (Lockwood *et al.*, 1998, 2007a; Nagase and Woessner, 1999). Associated with the reduced expression of MMPs is down-regulation of PAs. The combined effects of these complementary changes inhibit fibrinolysis to prevent hemorrhage, and further stabilize the endometrial ECM to promote vascular integrity (Lockwood *et al.*, 1995). Taken together, these coordinated progesterone-induced biochemical alterations create a local hemostatic endometrial environment that prevents potentially pregnancy terminating and life-threatening maternal hemorrhage during blastocyst implantation and placentation.

Menstruation is also mediated by HESCs

In non-fertile cycles, the premenstrual period of the late secretory (luteal) phase is accompanied by a decline in circulating progesterone levels, which triggers reductions in TF and PAI-1 expression (Lockwood *et al.*, 1994b) to create a pro-hemorrhagic and fibrinolytic milieu. Concomitantly, progesterone withdrawal initiates intense vasospasm linked to the increased biological availability of epithelial-derived endothelin-1 (ET-1) and stromal cell-derived prostaglandin (PG) $F_{2\alpha}$ synthesis, which elicit intense constriction of the spiral arterioles to produce hypoxia and generate vessel damaging reactive oxygen species (ROS) (Ohbuchi *et al.*, 1995; Marsh *et al.*, 1996; Salamonsen *et al.*, 1999; Maybin *et al.*, 2011b). In HESCs, declining progesterone levels also promote secretion of MMP-1, which preferentially degrades the fibrillar collagen MMP-3 which can initiate a local proteolytic cascade by degrading a wide array of ECM proteins and by activating the secreted forms of other MMPs, and PAs, which act in concert to promote sloughing of the functional endometrial layer by dissolution of the endometrial ECM during menstruation (Lockwood *et al.*, 1995, 1998; Salamonsen *et al.*, 1997; Schatz *et al.*, 1994, 1997). Consistent with changes in human endometrium (Marbaix *et al.*, 1992; Singer *et al.*, 2000), up-regulation of several MMPs in response to progesterone withdrawal, have also been reported in both macaque and mouse models of menstruation (Rudolph-Owen *et al.*, 1998; Kaitu'u *et al.*, 2005; Critchley and Saunders, 2009; Coudyzer *et al.*, 2013).

Endometrial post-menstrual repair and angiogenesis

Menstruation is followed by restoration of vascular integrity, angiogenesis and efficient endometrial repair (Okada *et al.*, 2014; Maybin and Critchley, 2015). While rising estradiol levels stimulate proliferation of endometrial glandular and luminal epithelial cells as well as HESCs, vascular repair and angiogenesis is mediated by thrombin and/or hypoxia/ROS generated-VEGF derived from HESCs (Lockwood *et al.*, 2002) and Ang-2, a pro-angiogenic vessel-branching and permeability factor (Albrecht and Pepe, 2003; Bonagura *et al.*, 2010) derived from human endometrial endothelial cells (HEECs) (Krikun *et al.*, 2000a). Simultaneously, the absence of progesterone and the presence of thrombin inhibit secretion of the blood vessel stabilizing and maturing agent, Ang-1 (Pfaff *et al.*, 2006; Thomas and Augustin, 2009), derived from HESCs (Krikun *et al.*, 2004). Other angiogenic factors are also induced in the endometrium during this period of endometrial repair. Specifically, in progesterone withdrawal, hypoxia and $PGF_{2\alpha}$ act via hypoxia inducible factor (HIF)-1α and nuclear factor kappa B (NF-κB) transcription factors to induce expression of IL-8, a potent angiogenesis mediator and primary neutrophil chemoattractant (Tecchio and Cassatella, 2014; Tecchio *et al.*, 2014) as well as adrenomedullin (AM), a pluripotent peptide member of the calcitonin gene peptide superfamily that induces vasodilation and proliferation of endothelial cells (Maybin *et al.*, 2011a). These changes are accompanied by elevated endometrial expression of

connective tissue growth factor (CTGF), a multifunctional growth factor and angiogenic agent expressed at high levels during wound repair and at sites of connective tissue formation (Igarashi *et al.*, 1993; Brigstock, 2002; Ivkovic *et al.*, 2003). Elevated CTGF mRNA and protein levels have been observed in post-menstrual endometrial epithelial cells and following treatment of endometrial epithelial cells with PGE_2, $PGF_{2\alpha}$ or hypoxia (Maybin *et al.*, 2012).

Endometriosis and uterine hemostasis

Endometriosis is an inflammatory, estrogen-dependent gynecological disorder characterized by the presence of viable endometrial tissue, aka ectopic endometrial implants, outside the uterine cavity (Giudice and Kao, 2004; Krikun *et al.*, 2008; Cakmak *et al.*, 2009; Uz *et al.*, 2011; Bourdel *et al.*, 2015). In the USA, endometriosis affects ~15% of all reproductive-aged women and 20–50% of infertile women (Krikun *et al.*, 2008; Bulun, 2009; Han and O'Malley, 2014). Ectopic endometrial tissues are detected primarily on the pelvic peritoneum and ovarian surface and rarely in the pericardium, pleura, lung parenchyma, and even the brain (Krikun *et al.*, 2008). The etiology of endometriosis reflects retrograde menstruation or coelomic metaplasia or differentiation of endometrial stem cells and/or embryonic duct remnants (Mai *et al.*, 1998; Starzinski-Powitz *et al.*, 2001; Taylor *et al.*, 2002; Slater *et al.*, 2005; Cakmak *et al.*, 2009). Multiple cellular and molecular mechanisms including increased endometrial cell adhesion, angiogenesis, inflammation, an impaired immune response, aberrant estrogen signaling and progesterone resistance as well as reduced apoptosis are involved in the development, growth and survival of endometriotic lesions (Aznaurova *et al.*, 2014; Taylor *et al.*, 2015). However, the current review focuses on the roles of TF and thrombin in the pathogenesis of this disease.

An enhanced local inflammatory response is a common finding in both ectopic and eutopic endometrial tissues (Kayisli *et al.*, 2007; Cakmak *et al.*, 2009; Uz *et al.*, 2011) with increased vascularization at the site of endometrial adhesion and subsequent invasion contributing to the survival and growth of ectopic endometrial implants. The role of TF as a well-defined inducer of angiogenesis (Ahamed and Ruf, 2004; Belting *et al.*, 2004; Leppert and Eisenreich, 2015) stimulated our laboratory to compare TF expression in eutopic endometrium obtained from control women and from women with endometriosis as well as in ectopic endometrial loci using immunohistochemistry and *in situ* hybridization analyses. These studies revealed a progesterone-mediated prominent increase in TF mRNA and protein expression in decidualized HESCs during the luteal phase with glandular and luminal epithelium exhibiting minimal constitutive TF expression in normal endometrium throughout the menstrual cycle (Lockwood *et al.*, 1993a, b; Schatz *et al.*, 2003). In contrast, enhanced TF expression is observed in eutopic and ectopic endometrial glandular epithelial cells derived from women with endometriosis (Krikun *et al.*, 2008). In addition to TF expression, thrombin receptors PAR-1 and PAR-2 were found to be highly up-regulated in the glandular epithelium of eutopic endometrium (Hirota *et al.*, 2005; Krikun *et al.*, 2008; Osuga *et al.*, 2012b). Thus, local thrombin generation associated with retrograde menstruation may mediate endometriotic nidation and/or persistence. Taken together, these observations strongly support involvement of TF, thrombin and its receptors in mediating local angiogenic and inflammatory responses in endometriotic lesions (Fig. 2).

Under physiological conditions, TF is not expressed by the endothelium (Duanmu *et al.*, 2011); however, endothelial cells of blood vessels in solid tumors and choroidal tissue in macular degeneration express endothelial TF (Contrino *et al.*, 1996; Hu and Garen, 2000, 2001). Our laboratory described the anomalous expression of TF by endothelial cells in endometriotic lesions (Krikun *et al.*, 2010b). The immunoconjugate molecule (Icon) is a complex of a mutated low-coagulation-inducing factor VII (fVII) domain that binds to TF with high affinity and an IgG1 Fc (fVII/IgG1 Fc) effector domain that activates a natural killer (NK) cell cytolytic response against TF-bearing endothelial cells (Hu *et al.*, 1999; Hu and Garen, 2001). In view of high levels of TF in endometriotic endothelial cells, we used Icon in an athymic mouse model of endometriosis as a potential novel endometriosis therapy. These animal studies revealed that Icon administration disrupts vascular structures in endometriotic loci without apparent toxicity, reduced fertility and/or subsequent teratogenic effects to abolish endometriotic implants (Krikun *et al.*, 2010b). The ability of Icon to eliminate pre-existing pathological vessels may provide a novel therapy in endometriosis. A recent study showed that the administration of ENMD-1068, a PAR-2 antagonist, significantly suppresses ectopic endometrial tissue growth in a mouse model revealing another potential novel therapy that targets TF and PAR signaling in treating endometriosis (Wang *et al.*, 2014).

Aberrant placentation: role of decidual hemorrhage, inflammation and thrombin generation

Clinical observations

A retrospective cohort study of a large Danish pregnancy registry conducted by Lykke and colleagues determined that first trimester vaginal bleeding was most strongly associated with early (28–31 weeks) PTB (odds ratio [OR] 2.98; 95% CI: 2.50–3.54) (Lykke *et al.*, 2010a). Lykke *et al.* also observed that such bleeding was associated with subsequent preterm premature rupture of the membranes (PPROM) (OR 1.18; 95% CI: 1.01–1.37), placental abruption (OR 1.48; 95% CI: 1.30–1.68), and severe pre-eclampsia (OR 1.25; 95% CI: 1.09–1.43). Prolonged vaginal bleeding for a minimum of 5 days in early pregnancy is associated with a 2-fold increase in risk of pre-eclampsia (North *et al.*, 2011). Moreover, Weiss *et al.* (2004) found that that subjects experiencing light vaginal bleeding during the first trimester are more likely to have pre-eclampsia, preterm delivery, and placental abruption, whereas heavy vaginal bleeding subjects are more likely to experience intrauterine growth restriction, PTB, PPROM, and placental abruption. All of these disorders have been linked to shallow placentation, decidual vasculopathy and acute atherosis (Brosens *et al.*, 2002, 2009; Veerbeek *et al.*, 2015). Thus, the findings by Lykke *et al.* (2010a) indicate that first trimester bleeding is a marker of a primary defect in placentation. This hypothesis is supported by the work of Salafia and colleagues who observed increased prevalence of decidual hemosiderin in patients with PTB at less than 32 weeks (196/462; 43%) compared with those with term birth (1/108; 0.8%) ($P < 0.00001$) (Salafia *et al.*, 1995). Moreover, decidual hemosiderin was also observed in 60% of patients with

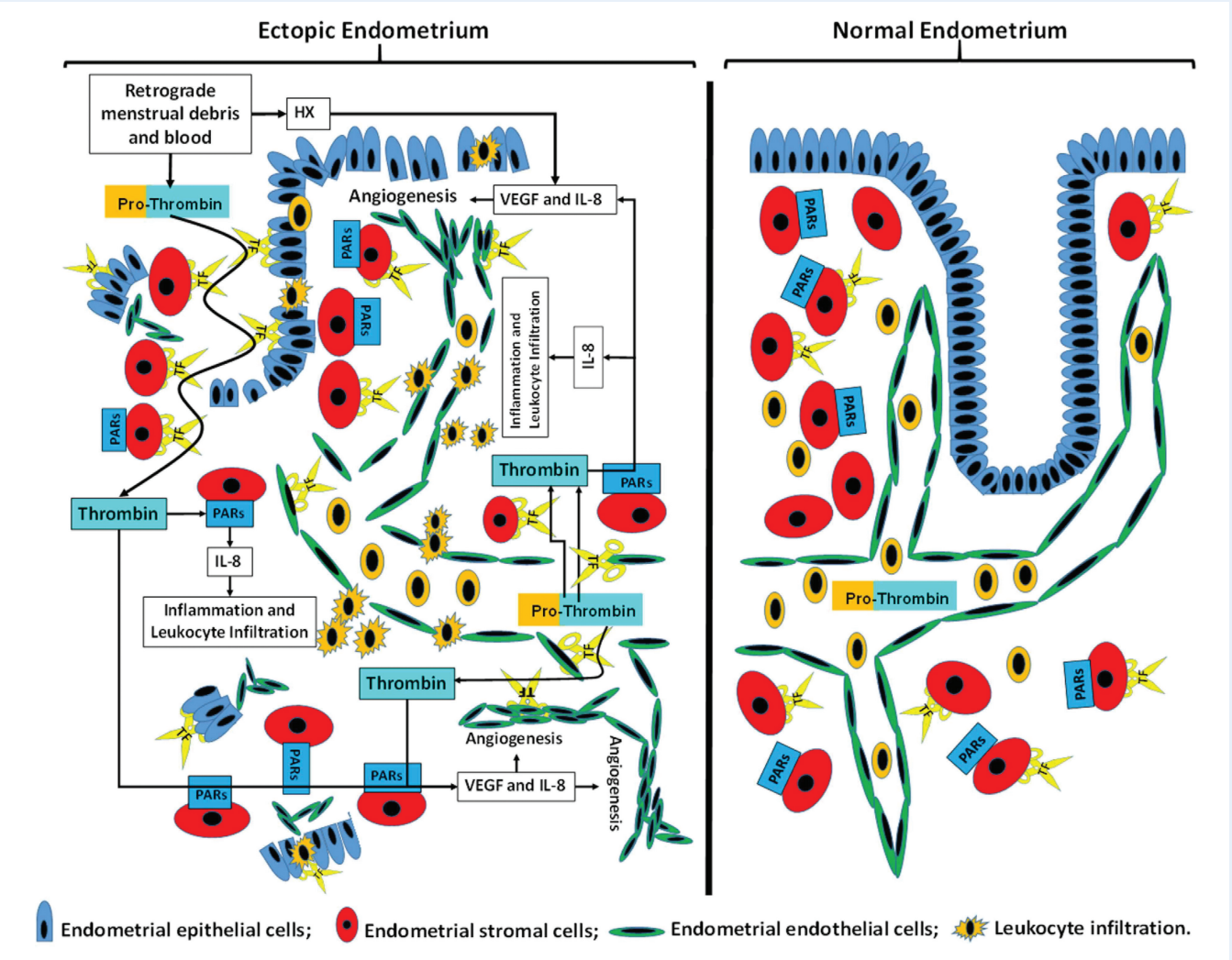

Figure 2 Role of tissue factor generated thrombin in the pathogenesis of endometriosis. Expression of tissue factor (TF) is limited to endometrial stromal cells in normal endometrium, whereas in endometriosis, in addition to endometrial stromal cells TF is also expressed by epithelial and endothelial cells. TF expressed by stromal and epithelial cells generates excess thrombin by cleaving prothrombin present in retrograde menstrual cell debris and blood. Additional prothrombin cleavage occurs by TF expressed by endometriotic endothelial cells. The resulting excess thrombin binds to Protease-Activated Receptors (PARs) in endometriotic stromal cells to induce thrombin-mediated signaling cascades that initiate secretion of several cytokines and growth factors, specifically IL-8 and VEGF. Both cytokines trigger endometriotic angiogenesis, and IL-8 also recruits leukocytes, dominated by neutrophils. During retrograde menstruation, hypoxia also promotes vascularization of endometriotic implants. TF: tissue factor/factor VII/VIIa.

preterm pre-eclampsia (45/76), in 36% of patients experiencing preterm labor with intact membranes (58/161) and in 38% of patients with PPROM (72/192). This study by Salafia and colleagues also found that first trimester vaginal bleeding was commonly associated with such decidual hemosiderin deposition, suggesting that decidual hemorrhage was the primary cause of first trimester bleeding.

Molecular mechanisms of aberrant placentation

While the precise etiology of the aberrant/shallow placentation associated with these adverse pregnancy outcomes remains uncertain, decidual hemorrhage at any gestational age triggers substantial local thrombin generation derived from the abundant levels of TF expressed on decidual cell membranes. However, during the first trimester, but not at term, thrombin binds to PAR1 and PAR3 to increase expression of soluble fms-like tyrosine kinase-1 (sFlt-1) by cultured human decidual cells (Lockwood *et al.*, 2007b). Other documented sources of enhanced sFlt-1 expression in pre-eclampsia include villous and extravillous trophoblasts (Tsatsaris *et al.*, 2003; Nagamatsu *et al.*, 2004; Fan *et al.*, 2014) as well as maternal peripheral mononuclear cells (Rajakumar *et al.*, 2005). A previous study showed that exogenous sFlt1 directly inhibits placental cytotrophoblast invasion *in vitro* (Zhou *et al.*, 2002). Recent *in situ* observations revealed that lower levels of sFlt-1 immunoreactivity in placental villi are associated with invasive placentation suggesting a critical functional role for sFlt-1 in regulating placental invasion (McMahon *et al.*, 2014). By irreversible binding to circulating VEGF, sFlt-1 blocks VEGF binding to its receptors on the endothelial cell membrane,

thereby mediating VEGF signaling and its physiologic actions (Powe *et al.*, 2011). During pregnancy, rodents overexpressing sFlt-1 develop hypertension, proteinuria and characteristic renal glomerular changes of endotheliosis that are clinical symptoms used to diagnose pre-eclampsia (Maynard *et al.*, 2003). Therefore, such thrombin-induced decidual cell expressed sFlt-1 may promote shallow placentation by impeding local angiogenesis and extravillous trophoblast (EVT) invasion during early pregnancy. The specificity of these thrombin effects is evident since neither tumor necrosis factor-α (TNF-α) nor IL-1β, cytokines established to be associated with pre-eclampsia, IUGR and shallow placentation (Rinehart *et al.*, 2001; Equils *et al.*, 2005; Zhou *et al.*, 2012; Matias *et al.*, 2015), enhanced sFlt-1 expression in cultured first trimester human decidual cells (Lockwood *et al.*, 2007b).

Previously, our laboratory demonstrated a statistically significant increase in macrophages (CD68-positive cells) and dendritic cells together with immunostaining of MCP-1, the primary monocyte recruiting and activating chemokine, in the decidua of preeclamptic patients (Lockwood *et al.*, 2006b). Consequently, activated macrophages are associated with EVT apoptosis and impaired migration promoting shallow EVT invasion and impaired spiral artery remodeling linked to pre-eclampsia, abruption, stillbirth, IUGR and many cases of spontaneous PTB (Guenther *et al.*, 2012; Melgert *et al.*, 2012; Faas *et al.*, 2014; Tang *et al.*, 2015). Moreover, thrombin enhances the secretion of MCP-1 by primary cultures of first trimester human decidual cells (Matta *et al.*, 2007). The pro-inflammatory cytokines, TNF-α and IL-1β also markedly increase MCP-1 secretion by cultured first trimester human decidual cells (Lockwood *et al.*, 2006b). In addition, we observed that IL-1β and TNF-α significantly increase secreted levels of the specific macrophage activator, macrophage colony stimulating factor (M-CSF) by first trimester decidual cells and that conditioned medium supernatants from these cultures activate macrophages, which then promote caspase 3/7-dependent EVT apoptosis (Wu *et al.*, 2012). Similarly, we observed increased expression of GM-CSF in pre-eclamptic decidua (Huang *et al.*, 2010) and that TNF-α and IL-1β each markedly increase GM-CSF production by cultures of primary first trimester human decidual cells. Finally, incubation with IL-1β markedly increases decidual cell expression of five additional chemokines responsible for monocyte/macrophage chemoattraction and activation: C-C motif ligand 2 (CCL2), CCL5, C-X-C motif ligand 2 (CXCL2), CXCL3 and CXCL8 (Huang *et al.*, 2006).

Role of immune cells in aberrant placentation

Several studies have shown the importance of regulatory T cells (Tregs) in physiopathology of pregnancy. During pregnancy, regulatory T cells (Tregs) play important roles in establishing and maintaining active immune tolerance (Aluvihare *et al.*, 2004; Du *et al.*, 2014). Tregs expand in the periphery and especially at the maternal-fetal interface in human and murine pregnancies. Moreover, the proportion of systemic and decidual Tregs (dTregs) was shown to be significantly lower in specimens from women with recurrent miscarriages compared with that in specimens from women with a normal pregnancy (Leber *et al.*, 2010). Several studies implicated their deficiencies in pregnancy complications such as pre-eclampsia, but the results are inconsistent among studies (Quinn and Parast, 2013; Rahimzadeh *et al.*, 2016). However, most recent studies suggest lack/reduced suppressive activity of Tregs in pre-eclampsia, associated with disruption of maternal immune tolerance (Hafeez *et al.*, 2014; Boij *et al.*, 2015; Nagayama *et al.*, 2015).

Complementing increased numbers of activated macrophages in the decidua of patients with pre-eclampsia is a reciprocal reduction in numbers of dNK cells (Lockwood *et al.*, 2013). In early human pregnancy, uterine spiral arteries and arterioles are converted to low resistance, high capacity vessels (conduits) that deliver increased blood flow required by the developing fetal-placental unit (Caniggia *et al.*, 2000; Huppertz, 2014a; Salomon *et al.*, 2014). This vascular conversion was initially attributed solely to fibrinoid-embedded blastocyst-derived EVTs (Robson *et al.*, 2012). Subsequent studies indicated that dNK cells, accumulated around spiral arteries in the absence of EVTs (Bilinski *et al.*, 2008; Male *et al.*, 2010), initiate such early steps in vessel remodeling as vascular dilation, endothelial hyperplasia and vacuolization of medial smooth muscle cells by expressing MMPs (Smith *et al.*, 2009), Interferon-γ (IFN-γ) and such angiogenic factors as VEGF-C and Ang-1 and Ang-2 (Ashkar and Croy, 2001; Kopcow and Karumanchi, 2007; Lash *et al.*, 2010). By recruiting circulating NK cells to the decidua, trophoblasts (EVTs) may indirectly affect early vascular remodeling (Greenwood *et al.*, 2000). Specifically, EVTs lining spiral arteries express stromal cell derived factor-1 (CXCL12), which binds to CCR4 expressed at the surface of the minority $CD56^{bright}CD16^{-}$ circulating NK cell population to promote their recruitment (Hanna *et al.*, 2003).

Studies in our laboratory revealed that co-incubation of primary cultures of human first trimester decidual cells with IFN-γ, a primary $CD56^{bright}16^{(-)}$ NK cell product and either macrophage-derived TNF-α or IL-1β synergistically enhances mRNA and protein expression of IFN-γ induced protein 10 (IP-10) and IFN-inducible T-cell-α chemoattractant (I-TAC) via Janus kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) and NF-κB signaling pathways (Lockwood *et al.*, 2013). Both chemokines are documented to recruit CXCR3-expressing NK cells (Gasperini *et al.*, 1999). Consistent with this *in vitro* action, immunostaining of first trimester decidua showed expression of IP-10, I-TAC, IFN-γ receptor 1 and 2, which are required to mediate the actions of IFN-γ on decidual cells (Chen *et al.*, 2015).

Flow cytometry identified elevated CXCR3 expression on dNK cells and on the minority of circulating peripheral $CD56^{bright}CD16^{-}$ NK cells and intermediate CXCR3 expression on the majority circulating $CD56^{dim}CD16^{+}$ NK cells. Peripheral NK cells incubated with either IP-10 or I-TAC displayed a concentration-dependent increase in CXCR3 levels and enhanced NK cell migration that peaked at 10 ng/ml. However, higher IP-10 and I-TAC concentrations (>50 ng/ml) elicited down-regulation of NK cell CXCR3 expression and migration. Consistent with this biphasic *in vitro* action, decidua derived from women with pre-eclampsia displayed significantly lower NK cell numbers and higher IP-10 and I-TAC expression compared with gestational age-matched controls. Consistent with these observations, we found significantly elevated IP-10 levels in first trimester sera from women destined to develop pre-eclampsia (Lockwood *et al.*, 2013). A recent study from our laboratory demonstrated that first trimester decidual cells are also a significant source of IFN-γ (Chen *et al.*, 2015). Thus, immunostaining of first trimester decidual sections revealed that both decidual cells and dNK cells express IFN-γ. However, while individual NK cells express higher levels of IFN-γ, the more numerous decidual cells account for a greater proportion of total decidual IFN-γ immunostaining. These findings suggest a 'Goldilocks' phenomenon in which some inflammation is required for optimal dNK and EVT remodeling of decidual and myometrial spiral arteries to increase uteroplacental blood flow, but excess inflammation may promote shallow placentation.

The cytokine, IL-11 is also a hemopoietic growth factor that exhibits pleiotropic biological effects, including induction of T helper cell type 2 (Th2) and inhibition of T helper cell type 1 (Th1) cytokine responses as well as induction of decidua-specific maturation of NK cells (Quesniaux *et al.*, 1992; Weich *et al.*, 1997; Bozza *et al.*, 2001; Curti *et al.*, 2001; Cakmak *et al.*, 2005), indicating complex regulatory effects in controlling inflammation. Acting as an anti-inflammatory agent, IL-11 reduces TNF-α levels in endometrial epithelial cells (Cork *et al.*, 2002) as well as IL-1β, TNF-α, IL-12, and nitric oxide production in activated macrophages (Trepicchio *et al.*, 1996) and CD4+ T cell production of T helper cell type 1 cytokines, such as IL-12 induced IFN-γ, while enhancing the expression of Th2 cytokines, such as IL-4 and IL-10 (Bozza *et al.*, 2001; Curti *et al.*, 2001). However, its well-documented pro-inflammatory properties include induction of PGE_2 secretion during bone resorption (Morinaga *et al.*, 1998; Morgan *et al.*, 2004) and stimulation of the expression of several acute phase proteins, including fibrinogen, C-reactive protein, ferritin, and haptoglobin (Gordon *et al.*, 1996; Morinaga *et al.*, 1998). In addition to changes in IP-10 and ITAC expression, we detected a pre-eclampsia-related increase in IL-11 levels in human decidual cells, but no changes in interstitial trophoblast at the maternal-fetal interface (Basar *et al.*, 2010). In parallel with these findings, IL-1β and TNF-α significantly induced levels of IL-11 mRNA expression and protein secretion in first trimester decidual cell cultures. Both the IL-1β- and TNF-α-mediated increases in IL-11 levels were blunted by specific inhibitors of the p38 mitogen-activated protein kinase (MAPK) and NF-κB, but not by protein kinase C signaling pathways (Basar *et al.*, 2010). As noted above, the role of IL-11 in regulating Th2 and Th1 cell activity and maturation of dNK cells as well as the complex involvement of IL-11 expression with inflammation, decidualization and trophoblast invasion suggests that enhanced expression of IL-11 contributes to the pathogenesis of pre-eclampsia by impairing dNK cell-mediated induction of endovascular EVT invasion of the decidua, the primary placental defect of pre-eclampsia.

Previously, our laboratory demonstrated higher immunostaining for MMP-9 in decidual cells of preeclamptic compared with gestational age-matched control placentas and that TNF-α and IL-1β each induce expression of MMP-9 mRNA and protein in cultured first trimester decidual cells (Lockwood *et al.*, 2008). We also observed that p38 MAPK signaling mediates TNF-α enhancement of first trimester decidual cell MMP-1, MMP-3, and MMP-9 expression (Lockwood *et al.*, 2014). These MMPs display both individual and overlapping ECM-degrading activities. Specifically, interstitial collagenase (MMP-1) preferentially degrades interstitial and fibrillar collagens, and stromelysin (MMP-3) degrades a broad array of proteins and can initiate a proteolytic cascade by activating the secreted zymogenic form of other MMPs such as pro-MMP-1 and pro-MMP-9, a collagenase (Itoh and Nagase, 1995; Westermarck and Kahari, 1999; Lijnen, 2002; Cohen *et al.*, 2006; Nagase *et al.*, 2006). During placentation, an inflammatory cytokine-induced MMP overexpression initiates a proteolytic cascade that disrupts the decidual ECM structure/integrity to interfere with normal stepwise integrin-mediated EVT invasion of the decidua and associated spiral artery and arteriole remodeling (Damsky and Fisher, 1998; Lyall, 2005; Pijnenborg *et al.*, 2006). Unexpectedly, our laboratory observed that IFN-γ inhibits this TNF-α enhancement of decidual cell p38 MAPK phosphorylation to block increased MMP-1, 3, and 9 expression, thereby stabilizing the decidual ECM to protect against pre-eclampsia (Lockwood *et al.*, 2014). Taken together, these findings also suggest the existence of a delicate balance that enables decidual cell and dNK cell-derived IFN-γ to synergistically interact with low levels of decidual macrophage-derived TNF-α to chemoattract peripheral $CD56^{bright}/CD16^{-}$ NK cells to the decidua. These newly recruited dNK cells can then release additional IFN-γ to promote EVT invasion, stabilize the decidual ECM and facilitate spiral artery vascular remodeling. Supporting these observations, IFN-γ deficient pregnant mice fail to display characteristic mid-gestational decidual vascular dilation and loss of smooth muscle. However, treatment with IFN-γ ameliorates these effects and mediates transformation of uterine spiral arteries to low resistance, high capacity vessels (Monk *et al.*, 2005). In contrast to this physiological state, pre-conceptional or peri-conceptional decidual inflammatory states associated with increased TNF-α or IL-1β generation would predictably upset this delicate balance to recruit to the decidua activated monocyte/macrophages whose elaboration of excess TNF-α causes EVT apoptosis, restrains dNK cell chemotaxis and disrupts decidual ECM to promote shallow placentation associated with various adverse pregnancy outcomes. Such a decidual inflammatory state could be caused by a variety of potential mechanisms including nulliparity-associated exaggerated alloimmune responses, autoimmune dysfunction, anti-phospholipid antibodies, severe obesity, chronic microbial infections and/or excess decidual thrombin generated by focal hemorrhage or severe thrombophilic conditions (e.g. anti-thrombin deficiency).

Decidual hemorrhage and inflammation are major contributors to preterm birth

Clinical observations

The occurrence of decidual hemorrhage and inflammation in the first trimester of pregnancy are strongly linked to aberrant placentation (Orsi and Tribe, 2008; Huppertz, 2014b). Moreover, bleeding into the decidua (i.e. abruption) (Naeye, 1983; Salafia *et al.*, 1995; Ananth *et al.*, 2007) and the occurrence of decidual inflammation later in pregnancy are strongly associated with spontaneous PTB (Romero *et al.*, 1988, 2002; Yoon *et al.*, 2001; Shim *et al.*, 2004). These two phenomena are linked since abruption (i.e. bleeding into the decidua basalis) is commonly (58%) associated with incomplete spiral artery transformation (Oyelese and Ananth, 2006). Abruption may present as vaginal bleeding, retrochorionic hematoma formation or a concealed abruption. The latter, if undermining >50% of the overlying placenta, can result in fetal death (Oyelese and Ananth, 2006). Vaginal bleeding in more than one trimester is associated with a 7-fold increased risk of PPROM and PTB compared with the absence of bleeding (Harger *et al.*, 1990), complementing the particularly strong association between abruption and PTB due to either PPROM or preterm labor with intact membranes between 22 and 32 weeks (Salafia *et al.*, 1995). Manuck and associates analyzed results from a multicenter, prospective study of 1025 women having singleton gestations with spontaneous PTB <34 weeks to categorize their clinical phenotype (Manuck *et al.*, 2015). This study reported decidual hemorrhage in 39% of women with very early (20–28 weeks) PTB, whereas decidual hemorrhage was present in 28% of cases of PTB between 28 and 34 weeks.

Abruptions are associated with significant thrombin generation manifested by the common occurrence of co-existent hypofibrinogenemia

and frank consumptive coagulopathy. Previously, our laboratory found that thrombin activation, as measured by elevated second trimester plasma thrombin-anti-thrombin (TAT) levels, predicts the subsequent occurrence of PPROM and PTB with high sensitivity and specificity (Elovitz *et al.*, 2001; Rosen *et al.*, 2001; Chaiworapongsa *et al.*, 2002).

Molecular mechanisms underlying thrombin-induced abruption

We have examined mechanisms underlying the strong association between decidual hemorrhage and PPROM and observed that thrombin-PAR interactions lead to the release of active MMP-1 and -3 by term decidual cells (Rosen *et al.*, 2002; Mackenzie *et al.*, 2004). Thrombin exhibits concentration-dependent direct weakening of isolated amniotic membranes and promotes amniotic MMP-9 release. All three MMPs are integral to fetal membrane rupture and cervical change (Fortunato *et al.*, 1999; Maymon *et al.*, 2000; Becher *et al.*, 2008; Zaga-Clavellina *et al.*, 2011). A recent study (Mogami *et al.*, 2014) revealed that thrombin activity is increased in PTB-derived human amnion and that thrombin treatment enhances MMP-1 and MMP-9 mRNA levels and enzymatic activity as well as cyclooxygenase 2 (COX-2) mRNA and PGE_2 production in amniotic mesenchymal, but not epithelial, cells. Moreover, this study localized PAR-1 to amniotic mesenchymal cells and decidual cells and demonstrated that thrombin-induced up-regulation of MMP-9 is specifically mediated by PAR-1, whereas thrombin enhanced MMP-1 and COX-2 expression is mediated via Toll-like receptor-4. In pregnant mice, thrombin injection induces PTB. Taken together, these observation indicate that thrombin acts through distinct mechanisms to activate MMPs and PGE_2 synthesis in amnion, thereby, contributing to PTB (Mogami *et al.*, 2014). Our group also demonstrated that abruptions are associated with a marked increase in decidual neutrophil infiltration accompanied by neutrophil co-localization with fibrin deposition, which peaks after PPROM. In contrast, decidua from gestational age-matched controls is virtually devoid of neutrophils. Moreover, thrombin markedly enhances mRNA and protein levels of the primary neutrophil chemoattractant IL-8 (Bergin *et al.*, 2010) in term decidual cells (Lockwood *et al.*, 2005).

In sections of human decidua, we found that abruption-associated PTB is accompanied by a decrease in immunohistochemical staining for decidual cell progesterone receptor (PR) expression (Lockwood *et al.*, 2012). Treatment of primary third trimester decidual cell cultures with thrombin elicited a concentration-dependent reduction in the two nuclear PR protein isoforms, PR-A and B and PR mRNA levels, mediated by phosphorylation of the signal transduction mediator, ERK1/2. Moreover, immunostaining for phospho-ERK1/2 was increased in decidual cells derived from abruption versus control decidua. Abruption-associated-reduced PR-A and PR-B expression by decidual cells suggests a mechanism by which excess thrombin derived from decidual cell-expressed TF acts as an autocrine/paracrine inducer of functional progesterone withdrawal (Zakar and Hertelendy, 2007; Patel *et al.*, 2015) to elicit abruption-related PPROM and PTB (Fig. 3).

In the previously cited study by Manuck *et al.*, inflammation and infection were found to be common causes of PTB (Manuck *et al.* 2015). Their study observed that among women with early (28–34 weeks) and very early (20–28 weeks) PTB, clinical and/or histological evidence of chorioamnionitis was present in 35 and 47% of cases, respectively. Analogous to the parallel effects of thrombin and either, TNF-α or IL-1β on first trimester decidual cells leading to aberrant placentation, TNF-α and IL-1β are integral to chorio-decidual infection (Flores-Herrera *et al.*, 2012) with both cytokines exhibiting similar *in vitro* pro-parturition effects on third trimester human decidual cells as those displayed by thrombin. Moreover, as is the case for abruption-related PTB, decidual cell immunostaining for MMP-1 and -3 is greatly increased in chorioamnionitis-complicated preterm decidual sections compared with control sections (Oner *et al.*, 2008). In third trimester human decidual cell cultures, TNF-α significantly enhances MMP-1 and -3 release by 14 ± 3 and 9 ± 2-fold, respectively, while IL-1β exerts even greater effects by increasing MMP-1 and -3 release, 13 ± 3- and 19 ± 2-fold, respectively. All of these effects are mediated by activation of p38 MAPK signaling (Fig. 3). We also observed that while neither TNF-α nor IL-1β weakened isolated amniotic membranes, co-incubation of either of these cytokines with both amnion side (facing the fetal site) and choriodecidua side (facing the maternal side) or with full thickness fetal membranes resulted in weakened fetal membranes together with excess production by the choriodecidua of MMP-9. These findings underscore the crucial role of decidual cells in mediating inflammation-associated PPROM (Kumar *et al.*, 2011). Extending these observations, a recent study by Kumar *et al.* demonstrated that weakening of full-thickness fetal membrane fragments exposed to TNFα or thrombin is blocked by pre-incubation with a CSF-2 antibody. A concurrent elevation of CSF-2 levels on the choriodecidua, but not the amnion side suggest that CSF-2 is a critical common intermediate in both thrombin derived from decidual cell-expressed TF and TNF-α induced fetal membrane weakening (Kumar *et al.*, 2014).

Molecular mechanisms contributing to inflammation-induced preterm birth

As is the case with abruption-associated PPROM, chorioamnionitis-associated PTB is accompanied by intense neutrophil infiltration of the decidua. We found that decidua from chorioamnionitis-complicated pregnancies, but not from term controls, displayed marked immunohistochemical staining for IL-8, as noted above, the primary neutrophil chemoattractant (Rosenkilde and Schwartz, 2004; Dimberg, 2010; Gales *et al.*, 2013) in decidual cells accompanied by dense decidual neutrophil infiltrates (Lockwood *et al.*, 2006a). In third trimester leukocyte-free decidual cell cultures, TNF-α and IL-1β induced a respective 236.6 ± 51.4 and 1062.6 ± 254.3-fold release of IL-8 accompanied by a corresponding 10- and 100-fold increase in steady state IL-8 mRNA levels (Fig. 3). Similarly, our laboratory demonstrated a marked increase in immunostaining for CSF2, (aka GM-CSF), which is a potent neutrophil and monocyte/macrophage chemoattractant and activator (Castagnola and Dufour, 2014; Hansen *et al.*, 2014), in the decidua of patients with chorioamnionitis compared with controls (Arcuri *et al.*, 2009). Augmenting these *in situ* observations, human third trimester leukocyte-free decidual cells treated with TNF-α or IL-1β exhibited statistically significant 18- and 245-fold increases in CSF2 protein release, respectively, accompanied by parallel changes in CSF2 mRNA levels.

In contrast with the low neutrophil numbers in normal human decidua, neutrophils are the dominant immune cell type in the decidua in most cases of chorioamnionitis-induced PTB (Bry and Hallman, 1989; Presicce *et al.*, 2015). The maternal origin of these decidual neutrophils in cases of PTB associated with acute chorioamnionitis was demonstrated by fluorescence *in situ* hybridization for X and Y chromosomes (Steel *et al.*,

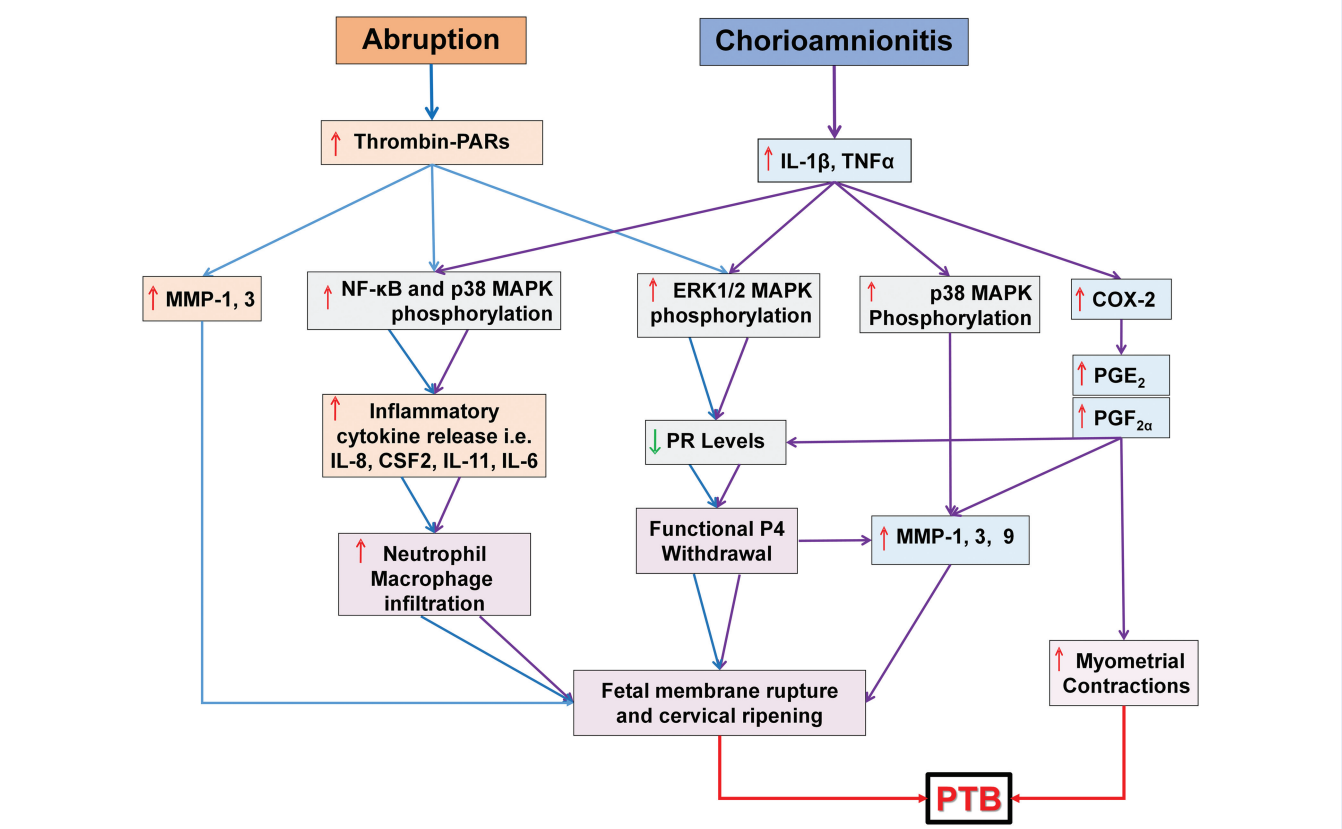

Figure 3 Cellular and molecular mechanisms leading to preterm birth. In the presence of decidual hemorrhage, thrombin generated from decidual cell-expressed TF activates NF-κB and p38 MAPK signaling pathways. Both pathways enhance production of several cytokines (IL-8, IL-11, CSF2, etc.), which recruit neutrophils that elaborate various matrix metalloproteinases (MMPs) as well as other proteinases. Thrombin directly enhances MMP-1 and -3 production in decidual cells. Collectively, these proteinases promote fetal membrane rupture and cervical change. Thrombin also suppresses decidual cell progesterone receptor (PR) levels by increasing ERK1/2 phosphorylation. The resulting functional progesterone withdrawal in the decidua further induces MMP expression and decidual inflammation. In the case of chorioamnionitis, pro-inflammatory mediators (IL-1β or TNF-α) and/or bacterial products (e.g. endotoxins) activate NF-κB, ERK1/2 and p38 MAPK mediated signaling pathways in decidual cells. NF-κB and p38 MAPK activation enhances production of cytokines (IL-8, IL6, IL11, etc.) and expression of MMP-1, -3, and -9 as well as cyclooxygenase (COX2), which in turn induce prostaglandins (PG) production in decidual cells. Moreover, the activated ERK1/2 MAPK signaling cascade lowers PR isoform levels causing functional progesterone withdrawal. Taken together, functional progesterone withdrawal, MMPs and increased PG production trigger uterine contractions, fetal membrane rupture and cervical ripening leading to chorioamnionitis-associated preterm birth (PTB).

2005). However, a subset of cases of chorioamnionitis linked to both neonatal intraventricular hemorrhage and death display a predominance of decidual monocytes/macrophages (Andrews *et al.*, 2006). Our laboratory demonstrated significantly higher decidual cell immunostaining for IL-6, a primary monocyte chemoattractant (Jones, 2005; Medzhitov, 2008; Schaper and Rose-John, 2015), in chorioamnionitis-associated PTB compared with gestational age-matched control placental specimens (Lockwood *et al.*, 2010). Levels of IL-6 were markedly increased in leukocyte-free term decidual cells by 2500-fold during incubations with IL-1β. Corresponding changes were also noted for steady state levels of IL-6 mRNA. Specific inhibitors of signaling for both NF-κB activation and p38 MAPK, but not for protein kinase C, inhibited this IL-1β inductive effect. Cervical IL-6 levels greater than 250 pg/ml between 24 and 36 weeks predict subsequent PTB with a sensitivity of 50.0% (95% CI: 33.2–66.8%) and a specificity of 85.0% (95% CI: 78.8–91.2%) (Lockwood *et al.*, 1994a), a predictive value only modestly inferior to fetal fibronectin (Lockwood *et al.*, 1991). These studies were extended to include IL-11, which is a key member of IL-6 cytokine family. Specifically, we observed significantly higher IL-11 immunostaining in decidual cells at the maternal-fetal interface obtained from women following preterm versus term delivery. These *in situ* observations were complemented by a marked increase in IL-11 production in term decidual cell cultures in response to thrombin or IL-1β treatment (Cakmak *et al.*, 2005). These findings suggest that aberrant IL-11 expression at the maternal-fetal interface is involved in inflammation- and abruption-associated prematurity since excess thrombin generation and IL-1β levels are associated with abruption- and chorioamnionitis-related preterm deliveries, respectively.

Analogous with abruption-associated PTB, immunostaining of human decidual sections identified significantly lower PR levels in decidual cells from chorioamnionitis-complicated PTB compared with gestational age matched controls (Guzeloglu-Kayisli *et al.*, 2015b). In correspondence with these *in situ* observations, treatment of third trimester leukocyte-free decidual cells with IL-1β significantly reduced PR mRNA and

protein expression while significantly enhancing decidual cell PGE_2 and $PGF_{2\alpha}$ production and COX-2 expression. We also found that $PGF_{2\alpha}$, but not PGE_2, suppressed decidual cell PR expression. However, the COX inhibitor, indomethacin, failed to reverse IL-1β inhibition of decidual cell PR expression, indicating that this cytokine directly inhibits PR expression, and therefore, that this suppression does not reflect a $PGF_{2\alpha}$ autocrine effect. While IL-1β treatment of decidual cells activated NF-κB, ERK1/2 and p38 MAPK signaling cascades, inhibition of ERK1/2 alone reversed IL-1β suppression of PR levels (Fig. 3). Thus, both thrombin and IL-1β inhibit expression of nuclear PR-A and PR-B to induce functional progesterone withdrawal via the same signal transduction pathway, demonstrating a parsimonious final common mechanism for abruption and infection-associated PTB (Lockwood *et al.*, 2012; Guzeloglu-Kayisli *et al.*, 2015b).

Abnormal uterine bleeding associated with pLARCs

Clinical observations

Administration of pLARCs to women results in safe, effective, and discrete long-term contraception (Affandi, 1998; Kaunitz, 2000; Rafie *et al.*, 2014). Currently available forms include: (i) Depo-Provera, an injectable form of medroxyprogesterone acetate (MPA) lasting 3 months; (ii) Mirena® intrauterine system (IUS) which releases levonorgestrel (LNG) for 5 years; (iii) Skyla IUS, a smaller version of Mirena with lower LNG content, ideally suited for nulliparous women and lasting over 3 years; and (iv) Nexplanon a single-rod etonogestrel contraceptive placed subdermally in the inner upper arm for 3 years (Varney and Guest, 2004; Chaovisitsaree *et al.*, 2005; Mutihir and Daru, 2008). Nexplanon is radio-opaque for ease of identification and removal. The extremely effective contraceptive properties of these agents reflect changes in cervical mucus and tubal motility that impair sperm migration, inhibition of gonadotrophin secretion to block ovulation, and, potentially, establishment of a hostile endometrial environment to prevent blastocyst implantation (Birgisson *et al.*, 2015). These agents are safe to use during lactation and are particularly appropriate to administer to women in whom estrogen therapy is contraindicated due to increased thromboembolic risks (Kaunitz, 2000). A large prospective cohort study in which more than 75% of teenage participants received pLARCs found a substantial reduction in mean annual rates of pregnancy, birth, and abortion compared with the overall pool of sexually experienced US teens (Secura *et al.*, 2014).

The occurrence of abnormal uterine bleeding in the majority of pLARC users is a major reason for non-adherence. Three year discontinuation rates were 47% for implant and 27% for IUS users, respectively (Weisberg *et al.*, 2014). Unlike the predictable and controlled nature of menstrual bleeding, pLARC-associated AUB occurs unpredictably and sporadically.

Cellular and molecular mechanisms underlying pLARC-induced abnormal uterine bleeding

We have biopsied bleeding and non-bleeding endometrial sites in pLARC versus secretory phase controls and observed that such AUB arises from superficial, enlarged, thin-walled fragile vessels denuded of pericytes and vascular smooth muscle cells, embedded in a collapsed stromal ECM (Runic *et al.*, 2000; Kayisli *et al.*, 2015). Unexpectedly, after 1 year of pLARC treatment, immunostaining demonstrated that TF levels are increased at endometrial bleeding versus non-bleeding sites. Equally surprising, bleeding sites exhibited high HESC immunoreactive PR and EGFR levels, suggesting that the molecular mechanisms responsible for pLARC-associated bleeding differed from those found in progesterone withdrawal triggered menstrual bleeding. This led us to examine the genesis of the abnormal endometrial vasculature found in these patients. We observed that pLARC treatment reduced endometrial blood flow up to 8-fold (Fig. 4) as measured by laser Doppler fluxmetry (Hickey *et al.*, 2006b). Studies in an animal model indicate that this effect is likely due to direct progestin-mediated vasoconstrictive effects on uterine or radial arteries (Krikun *et al.*, 2012). Compatible with this observation, pLARC treated endometria displayed evidence of hypoxia/reperfusion injury. Thus, women treated with the pLARC, Implanon, displayed increased endometrial immunostaining for the phosphorylated forms of the stress-activated kinases SAPK/JNK and p38 MAPK, markers of hypoxic injury (Krikun *et al.*, 2002). Consistent with these *in situ* observations, hypoxia and ROS induced phosphorylation of SAPK/JNK and p38 MAPK in primary cultures of both HESCs and HEECs. The endometria of pLARC users also display evidence of excess ROS generation (Fig. 4), as indicated by increased staining for nitrotyrosine and 8-hydroxydeoxyguanosine as well as evidence of increased 8-isoprostane production, a marker of lipid peroxidation (Hickey *et al.*, 2006b).

Consistent with the effect of hypoxia as the prime driver of angiogenesis (Hashimoto and Shibasaki, 2015; Schonenberger and Kovacs, 2015; Zimna and Kurpisz, 2015), histological sections of endometria from pLARC-treated patients display expected reduced immunostaining for Ang-1, as previously noted, a vessel stabilizing factor (Fukuhara *et al.*, 2010; Koh, 2013), and increased expression of the primary mediator of angiogenesis, VEGF, in HESCs with both changes correlating positively with microvascular density and increased bleeding profiles (Lau *et al.*, 1999; Roopa *et al.*, 2003; Lockwood *et al.*, 2004b; Pritts *et al.*, 2005). Simultaneously, increased immunostaining for Ang-2, a pro-angiogenic vessel-branching and permeability factor (Thurston and Daly, 2012; Eroglu *et al.*, 2013; Gerald *et al.*, 2013), was evident in HEECs (Lockwood *et al.*, 2004b). Furthermore, while MPA enhanced Ang-1 protein and mRNA expression, both hypoxia and ROS markedly decreased Ang-1 expression in HESCs (Krikun *et al.*, 2002). Conversely, HEECs maintained under hypoxia exhibited significantly increased Ang-2 expression (Fig. 4).

Excess thrombin is expected to be generated in response to pLARC-associated AUB. Thus, we incubated primary cultures of HESCs and human endometrial glandular epithelial cells (HEGECs) with MPA ± thrombin under hypoxia or normoxia. Compared with normoxia, hypoxia enhanced secreted levels of VEGF several-fold in HESCs or HEGECs, whereas MPA did not significantly affect VEGF output by either cell type under normoxia or hypoxia. Addition of thrombin to cultured HESCs elicited significant concentration and time-dependent increases in VEGF protein and mRNA that peaked at 48 h. Multiple VEGF isoforms were enhanced in HESCs by thrombin including VEGF-121, -165 and -189. By contrast, HEGECs were unresponsive to thrombin added either alone or with MPA. Extrapolation of these *in vitro* findings to endometria from pLARC treated women suggests that bleeding into decidualized HESCs that over-express TF generates thrombin, which in turn acts as an autocrine potentiator of VEGF expression

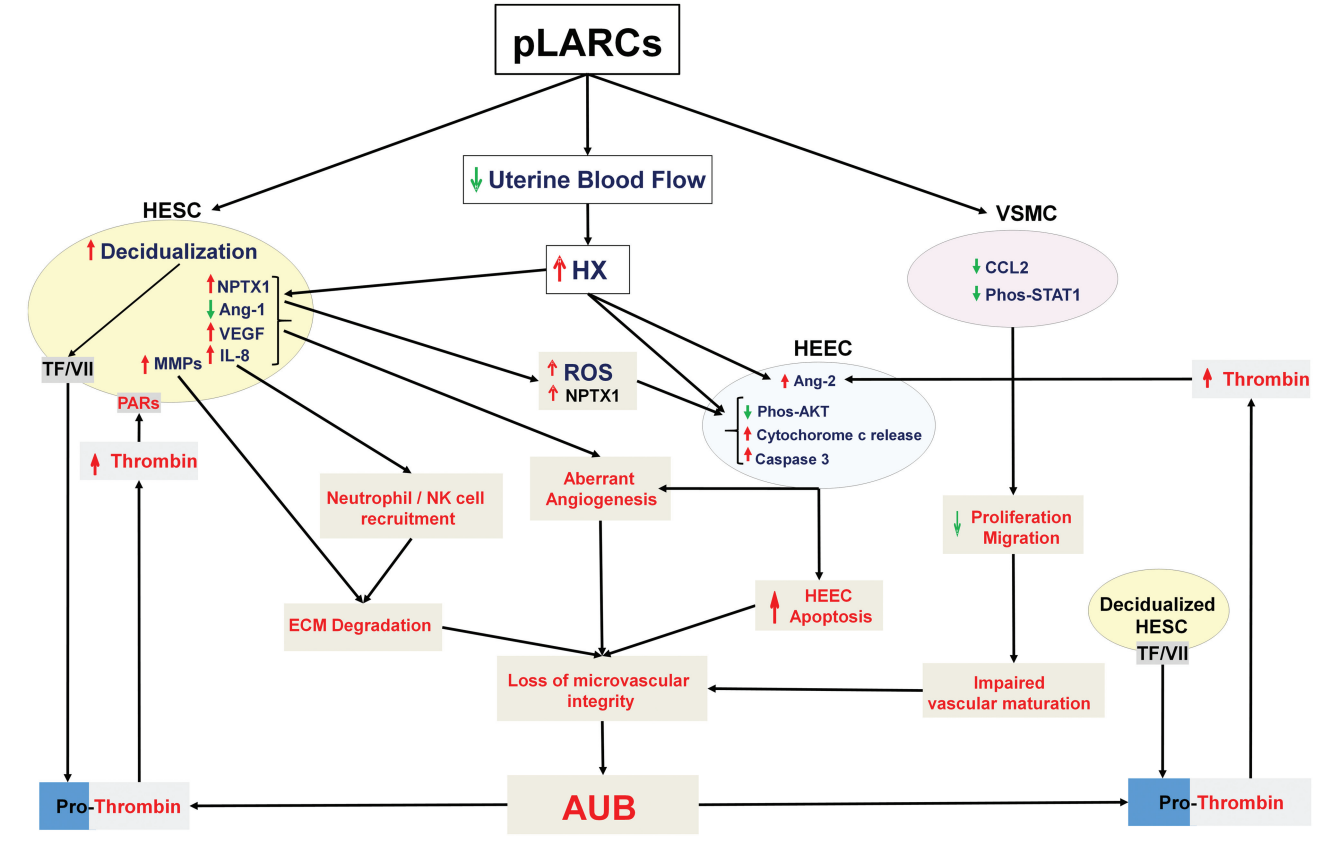

Figure 4 Cellular mechanisms of AUB in progestin-only, long-acting, reversible contraception (pLARC). Administration of pLARCs induces human endometrial stromal cell (HESC) decidualization and reduces endometrial blood flow causing local hypoxia. In decidualized HESCs, this hypoxia inhibits expression of angiopoietin (Ang)-1 while increasing expression of vascular endothelial growth factor (VEGF), interleukin (IL)-8 and matrix metalloproteinases (MMPs). In human endometrial epithelial cells (HEECs), hypoxia and ROS increase expression of Ang-2, a potent angiogenic agent. Hypoxia and reperfusion generate reactive oxygen species (ROS) that directly damages blood vessels, resulting in enhanced vascular permeability. The latter delivers circulating factor VII to HESC membrane bound tissue factor (TF), which activates factor Xa to generate thrombin. The resulting excess thrombin exacerbates hypoxia/ROS effects on endometrial angiogenesis and inflammation. pLARCs-induced hypoxia/ROS enhances HESC secreted NPTX1 which trigger HEEC apoptosis by inhibiting AKT signaling, causing mitochondrial dysfunction, enhancing cytochrome c release, and activating a caspase 3 mediated cascade. Moreover, pLARCs directly inhibit vascular smooth muscle cells (VSMC) proliferation and migration by blocking CCL2-mediated STAT1 signaling. The combined impact of these molecular mechanisms promotes aberrant angiogenesis and endothelial apoptosis, and reduces numbers of VSMCs to generate thin-walled hyperdilated fragile vessels prone to leakage and bleeding.

that exacerbates abnormal endometrial angiogenesis found in these women (Fig. 4) (Lockwood *et al.*, 2002).

To account for the fragility of endometrial microvessels in pLARC-administered women, we hypothesized that paracrine factors from pLARC-treated HESCs promote HEEC apoptosis. In support of this hypothesis, conditioned media supernatant (CMS) from HESCs treated with E_2+etonogestrel or E_2+MPA under hypoxia induced HEEC apoptosis (Guzeloglu-Kayisli *et al.*, 2014). Moreover, mass spectrometric analysis of this CMS revealed increased levels of neuronal pentraxin-1 (NPTX1), an apoptotic inducer (Clayton *et al.*, 2012; Al Rahim *et al.*, 2013; Thatipamula and Hossain, 2014). As expected, cleaved caspase-3 and NPTX1 immunoreactivity were significantly higher in the endometria from pLARC-treated women. Transcriptomic analysis of these cultures revealed inhibition of AKT signaling and mitochondrial dysfunction in HEECs incubated with HESC-derived CMS. We also found that incubation of HEECs with recombinant NPTX1 ± H_2O_2 increases both HEEC apoptosis and cytosolic cytochrome c levels. Therefore, in women, pLARC-enhanced NPTX1 secretion and ROS generation in HESCs would impair HEEC survival resulting in loss of vascular integrity leading to AUB (Fig. 4).

The endometrium of pLARC users contains excess neutrophils and NK cells (Hickey *et al.*, 2006a). We observed that Mirena contraception is associated with increased endometrial IL-8 immunoreactivity (Lockwood *et al.*, 2004a). In addition to acting as a well-documented potent chemoattractant for neutrophils, IL-8 also recruits NK cells (Loetscher *et al.*, 1996). Use of the discontinued pLARC, Norplant, was also accompanied by elevated expression by HESCs of the neutrophil activating chemokine, ENA-78 (Chegini *et al.*, 2007). Neutrophils express elastase and MMP-9, and NK cells express several MMPs that exhibit separate and common ECM-degrading specificities (Cundall *et al.*, 2003; Anacker *et al.*, 2011). The combined effects of these molecular changes help account for pLARC-associated AUB (Fig. 4).

Since the abnormal endometrial vasculature observed in pLARC users is characterized by thin-walled hyperdilated fragile microvessels prone to bleeding, we determined whether these vessels are associated with deficient pericytes and vascular smooth muscle cells (VSMCs) support (Kayisli *et al.*, 2015). Double-immunostaining for the presence of proliferating cell nuclear antigen (PCNA) and α-smooth muscle actin (α-SMA) was used to evaluate VSMC differentiation and proliferation respectively in endometria obtained from women before and after pLARC administration. These endometrial vessels displayed significantly reduced α-SMA immunoreactivity and fewer PCNA positive nuclei among α-SMA positive cells. Microarray analysis of primary cultures of human VSMCs treated with either MPA or etonogestrel demonstrated multiple pLARC-regulated genes including decreased CCL2. Of relevance to pLARC induced AUB, while both MPA and etonogestrel reduced VSMC proliferation and migration, recombinant CCL2 reversed this progestin-mediated inhibition. Thus, pLARC appears to inhibit endometrial pericyte and VSCM numbers by inhibiting CCL2 (Fig. 4).

The FK506-binding proteins (FKBP5) belong to a family of immunophilins that mediate actions of specific immunosuppressive drugs. Two members, FKBP51 and FKBP52, share 70% similarity and contain an active peptidyl-prolyl-isomerase domain as well as a tetracopeptide repeat domain that binds to the C-terminus of Hsp90 (Erlejman *et al.*, 2014; Mustafi *et al.*, 2014). This binding enables them to act as co-chaperones with Hsp90 in steroid receptor complexes. While FKBP52 positively regulates the PR, glucocorticoid receptor (GR) and androgen receptor, FKBP51 is a negative steroid receptor regulator (Riggs *et al.*, 2004; Cioffi *et al.*, 2011; Storer *et al.*, 2011; Sanchez, 2012). Overexpression of FKBP51 and PR in human HepG2 cells, decreases progestin responsiveness, suggesting the existence of a negative feedback loop (Hubler *et al.*, 2003). The observation that pLARC treatment does not decrease PR expression in HESCs led us to hypothesize that such treatment increases FKBP51 levels in HESCs, thereby setting into motion a negative feedback loop that impairs the responsiveness of crucial genes to progestin. We recently tested this hypothesis by performing FKBP51 immunohistochemistry on paraffin sections of endometria from women before (pre-) and after three months (post-) Depo MPA (DMPA) administration. In immunostained sections, FKBP51 HSCOREs were significantly higher in endometrial stromal and glandular cells from post-DMPA versus pre-DMPA (Guzeloglu-Kayisli *et al.*, 2015a). To complement this *in situ* finding in human endometrium, we performed immunostaining on endometrial sections from ovariectomized guinea pigs treated with estradiol or estradiol+MPA for 21 days. In the guinea pigs, estradiol +MPA significantly increased FKBP51 immunoreactivity in endometrial stromal and glandular cells versus estradiol-treatment. Moreover, ingenuity pathway analysis of microarray results from HESC cultures treated with MPA or etonogestrel identified GR activation as an upstream regulator of both MPA and etonogestrel-specific differentially regulated genes, which includes significant enhancement of FKBP51 (Guzeloglu-Kayisli *et al.*, 2015a). Moreover, compared with estradiol, estradiol+MPA treatment significantly inhibited IL-1β mRNA levels in control vector transfected HESCs, whereas MPA failed to inhibit IL-1β mRNA levels in HESCs overexpressing FKBP51. Furthermore, we found that in the presence of MPA, FKBP51 overexpression exacerbates thrombin-induced IL-1β mRNA levels in HESCs (Guzeloglu-Kayisli *et al.*, 2015a). Consequently, chronically increased endometrial FKBP51 expression in pLARC users due to GR signaling activation could contribute to AUB by inducing a negative feedback loop that inhibits PR and GR-mediated transcription. The resultant PR and/or GR-mediated functional withdrawal may contribute to associated endometrial inflammation, aberrant angiogenesis, and bleeding.

Conclusions

Perivascular decidualized HESCs promote endometrial hemostasis during placentation through progestational regulation of hemostatic (TF and PAI-1), anti-fibrinolytic and anti-protease activity, yet paradoxically facilitate menstruation following, progesterone withdrawal via elaboration of fibrinolytic, proteolytic, and vasoconstricting proteins in the setting of reduced TF and PAI-1 expression. Early in pregnancy, the pathological occurrence of decidual hemorrhage is linked to excess local thrombin generation, which contributes to adverse pregnancy outcomes including subsequent pre-eclampsia through multiple biochemical mechanisms including elaboration of sFlt-1 and MMPs, while modifying chemokine expression to enhance numbers of activated macrophages, while inhibiting dNK cell migration. The combined effects of these actions impede spiral artery transformation. These thrombin-induced effects on MMP and chemokine expression are mirrored by TNF-α and to lesser extent by IL-1β, linking inflammatory conditions to pre-eclampsia, and to other adverse pregnancy outcomes. Later in pregnancy, the excess production of these same mediators to cause PTB. Thus, decidual hemorrhage (abruption) leads to excess thrombin generation, which enhances decidual cell MMP and neutrophil infiltration and down-regulates PR expression to preferentially promote PPROM. Similarly, chorioamnionitis-associated IL-1β and to a lesser extent TNF-α, also induce MMPs, inhibit decidual PR expression and also increase COX-2 and PG production to promote PTB due to PPROM and/or preterm labor.

Finally, AUB associated with pLARC use is triggered by unexpected elevation in TF expression at specific bleeding sites accompanied by excess thrombin generation. This increased TF expression is accompanied by reduced endometrial blood flow that promotes hypoxia and ROS-driven aberrant angiogenesis and inflammation as well as progestin induced impairment in pericyte and VSMC envelopment of superficial endometrial vessels. Hypoxia elicits the elaboration of endothelial cell apoptotic inducers secreted by HESCs. The resultant hyper-dilated, thinned wall fragile vessels with disrupted endothelia are the source of AUB leading to high rates of discontinuation of these otherwise safe and effective contraceptives.

Future directions

Worldwide, the PTB rate is 5–18%, accounting for over 15 million births per year (Romero *et al.*, 2014). While the past decade has witnessed an 11% decline in US PTB rates to 11.4% (Schoen *et al.*, 2015), this condition remains the leading cause of perinatal morbidity and mortality in the USA, as well as a major antecedent of chronic lung disease, and neurodevelopmental disorders, accounting for $26 billion/year in health care costs (Rubens *et al.*, 2014). Despite significant research efforts, there has been only modest progress in elucidating underlying mechanisms. Studies focusing on identification of biomarkers are needed for early diagnosis of pregnancy complications leading to PTB. Similarly, little progress has been made in reducing the rate of pLARC-induced AUB. Significant reduction in the rates of PTB or pLARC-induced AUB requires further studies that focus on developing therapeutic agents that block molecules,

receptors and/or associated signaling pathways mediating PTB or pLARC-induced AUB. Progress in both areas requires integration of observations made in women with those obtained from relevant animal studies. While the potential contribution of specific transgenic mice is unquestioned, as previously demonstrated by our laboratory (Krikun *et al.*, 2010a, 2012; Kayisli *et al.*, 2015), the guinea pig is a particular appropriate model with which to evaluate reproductive system and pregnancy complications. Thus, like human endometrium, the guinea pig endometrium displays closely related human features such as spontaneous estrus cycling and hemochorial placentation (Krikun *et al.*, 2011).

Authors' roles

F.S., O.G.-K., S.A., U.A.K., and C.J.L. contributed equally to manuscript writing and approved the final version.

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Conflict of interest

None declared.

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