The Epidermal Growth Factor Receptor in Healthy Pregnancy and Preeclampsia

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Abstract

The epidermal growth factor receptor (EGFR) is expressed robustly in the placenta, and critical processes of pregnancy such as placental growth and trophoblast fusion are dependent on EGFR function. However, the role that aberrant EGFR signaling might play in the etiology and/or maintenance of preeclampsia (PE) remains largely unexplored. Recently, we have shown that overexpression of EGFR in cultured uterine artery endothelial cells (UAEC), which express little endogenous EGFR, remaps responsiveness away from vascular endothelial growth factor receptor (VEGFR) signaling and towards EGFR, suggesting that endothelial EGFR expression may be kept low to preserve VEGFR control of angiogenesis. Here we will consider evidence for the possibility that the endothelial dysfunction observed in PE might in some cases result from elevation of endothelial EGFR. During pregnancy, trophoblasts are known to synthesize large amounts of EGFR protein, and the placenta regularly releases syncytiotrophoblast (STB)-derived exosomes and microparticles into the maternal circulation. Although there are no reports of elevated *EGFR* gene expression in preeclamptic endothelial cells, the ongoing shedding of placental vesicles into the vascular system raises the possibility that EGFR-rich vesicles might fuse with endothelium, thereby contributing to the symptoms of PE by interrupting angiogenesis and blocking pregnancy-adapted vasodilatory function.

Keywords

EGFR; exosome; endothelium; preeclampsia; pregnancy

Introduction

Preeclampsia (PE) is a hypertensive disease of pregnancy that is both a leading cause of maternal morbidity and mortality worldwide, and confers an increased risk for developing

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cardiovascular diseases later in life (reviewed by Lee & Tubby 2015). To date, nothing can definitively prevent PE, and the only 'curative' treatment is still delivery of the baby and placenta. Attempts to map PE-specific patterns of placental gene expression have identified at least three major subclasses of PE, each with its own unique expression profile for known PE markers (Leavey et al. 2015). Significant progress has been made, however, in understanding the mechanisms of normal vascular adaptation to pregnancy, which comprises early angiogenic events and enhancement of pro-vasodilatory signaling, facilitated by the augmentation of connexin 43 (Cx43) gap junction-mediated intercellular $Ca2+$ signaling (Yi et al. 2010). We have previously pointed out that PE is associated with alterations in both growth factors and cytokines in a manner reminiscent of 'wounding' responses, and these same factors bring about loss of pregnancy-adapted Cx43 function and vasodilation (reviewed in Bird et al. 2013). While growth factors and cytokines are indeed essential for the establishment of pregnancy and the continuing development of the fetus, they require precise regulation to avoid the pathologies of pregnancy that result from deficient or excessive endocrine and paracrine signaling.

Some growth factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), have been studied extensively in the context of PE, but the role that altered epidermal growth factor (EGF) receptor signaling might play in promoting PE remains comparatively unexamined. Although accumulating data in rodent models suggest that aberrant EGFR signaling is likely a factor in the development and/or maintenance of general hypertensive diseases (Florian & Watts 1999; Sambhi et al. 1992; Kagiyama et al. 2002, 2003; Kim et al. 2006; Ying & Sanders 2005; Kelly et al. 2017), there is little published research on EGFR signaling in the context of hypertensive disease during pregnancy, probably because EGFR is not considered a major regulator of normal angiogenesis or vasodilatory function in either uterine or umbilical endothelium. Nonetheless, transcriptome meta-analysis has suggested that altered gene expression of *EGFR* and/or its ligands may be a factor in the impaired placental function associated with PE (Moslehi et al. 2013), but there has been no direct evidence to support changes in endothelial cell *EGFR* expression being associated with endothelial dysfunction in PE.

Interestingly, *EGFR* is generally expressed at low levels in 'normal' endothelial cells. In the vascular endothelium, the VEGF receptor (VEGFR) system is considered the key regulator of angiogenesis (reviewed in Ferrara & Davis-Smyth 1997), while the fibroblast growth factor receptor (FGFR) system is the key mediator of mitogenesis (Lindner et al. 1990). Recently, we have shown that artificial increases in EGFR expression in cultured uterine artery endothelial cells (UAEC) leads to a shift in responsiveness away from VEGFR and towards EGFR, suggesting that endothelial EGFR expression may normally be low to avoid interference with VEGFR control of angiogenesis (Clemente et al. 2020). Indeed, the purpose of this review is to now explore the evidence that endothelial dysfunction observed in PE might indeed result from elevation of endothelial EGFR through exosomal transfer from the PE placenta.

During a healthy pregnancy, trophoblasts are known to synthesize large amounts of EGFR protein (Uhlén et al. 2015), and the placenta releases syncytiotrophoblast (STB)-derived exosomes and microparticles into the maternal circulation (reviewed in Tannetta et al.

2017). The plasma concentration of placenta-derived exosomes has also been shown to rise markedly in each subsequent trimester (Salomon et al. 2014), and this is even greater in the third trimester in a subset of PE cases (Pillay et al. 2016). Although there are no reports of elevated *EGFR* gene *transcript* expression in preeclamptic endothelial cells, the ongoing shedding of placental vesicles into the vascular system raises the possibility that EGFR-rich vesicles might be taken up by the endothelium, promoting the symptoms of PE by interrupting angiogenesis and blocking pregnancy-adapted vasodilatory function.

Before proposing a new model for placental EGFR-mediated endothelial dysfunction, this review will (1) provide a brief overview of EGFR signaling in general, (2) review ways in which EGFR is critical for placental function and embryonic and fetal development, (3) cite the limited and contradictory data on altered EGFR expression and activity in PE, and (4) examine preliminary evidence suggesting that improperly regulated EGFR signaling can have deleterious effects on pregnancy-adapted vasodilation.

It should be noted that the term "EGFR signaling" should only be interpreted to mean "any signaling event that involves EGFR," and *not* "signaling performed exclusively by EGFR." Effector molecules that are activated by EGFR in one cellular environment may also be mimicked by another receptor (e.g. VEGFR2, FGFR1) in a different setting.

The epidermal growth factor receptor

EGFR is a 170 kDa transmembrane protein expressed in a wide variety of cell types. It is one of four members of the ErbB family of receptor tyrosine kinases comprising EGFR (ErbB1), HER2 (ErbB2), the kinase-impaired ErbB3, and the EGFR-like ErbB4. In healthy cells, EGFR sits dormant in the plasma membrane as a monomer (Yamashita et al. 2015) or inactive symmetric dimer (Jura et al. 2009) until it is activated by binding with one of seven known ligands, including EGF, heparin-binding EGF-like growth factor (HBEGF), and transforming growth factor alpha (TGFα) (Wilson et al. 2009). Binding of ligand to EGFR induces a conformational change in its transmembrane domain that releases EGFR from its intrinsically disordered basal inactive state (Shan et al. 2012), so allowing the formation of active asymmetric dimers (Zhang et al. 2006; reviewed in Purba et al. 2017). This asymmetric dimerization stimulates EGFR's tyrosine kinase activity, which results in the autophosphorylation of several tyrosines in the receptor's C-terminal tail, and subsequently receptor internalization. These phosphotyrosine residues serve as docking sites for a diverse array of effector molecules that initiate signaling cascades along major pathways, including PI3K/Akt, MAPK, JAK-STAT, and PLCγ (reviewed in Wee & Wang 2017; Jones & Rappoport 2014). In some cellular environments, EGFR can also phosphorylate tyrosine residues of other receptors, and be transactivated by G-protein coupled receptor (GPCR) agonists (Daub et al. 1996).

Under normal conditions, EGFR plays key roles in cell proliferation, tissue homeostasis, organismal growth and development, wound healing, immune function, and embryogenesis (reviewed in Wee & Wang 2017). However, precisely because of its critical importance in so many biological processes, the consequences of aberrant EGFR signaling can be deadly. Deficient EGFR signaling is associated with skin disorders, inflammatory bowel disease,

and improper cartilage formation leading to osteoarthritis (Brooke et al. 2014, Jia et al. 2016), while excessive EGFR signaling due to overexpression or activating mutation drives the progression of many cancers, including aggressive cancers of the breast, ovary, prostate, lung, colon, and brain (reviewed in Normanno et al. 2006).

EGFR in healthy pregnancy

Expression and localization of EGFR and its ligands in a healthy pregnancy

The critical importance of EGFR in pregnancy is immediately suggested by its high level of expression in the placenta, which far exceeds the levels observed in all other organs (Uhlen et al. 2015). Placental expression of EGFR varies in localization over the course of pregnancy, as does expression of EGFR ligands. Immunohistochemical staining of human placental tissues from weeks 4 and 5 of the first trimester showed that almost all expression of EGF and EGFR was localized in cytotrophoblasts (CTBs). During weeks 6 to 12, however, both EGF and EGFR were localized primarily in STBs (Ladines-Llave et al. 1991), suggesting that the role of placental EGFR changes during different stages of fetal development. Other EGFR ligands, including amphiregulin (AREG) (Lysiak et al. 1995) and betacellulin (BTC) (Tanimura et al. 2004), are also expressed in the placenta in a compartmentalized and time-dependent manner. TGFα is moderately expressed and HBEGF is robustly expressed in 1st trimester decidua and in CTBs and extravillous trophoblasts (EVTB) during all three trimesters (Lysiak et al. 1993; Birdsall et al. 1996; Leach et al. 1999). TGFα is also expressed in the STB during all three trimesters (Lysiak et al. 1993), but HBEGF expression is poor in the STB during the first trimester (Yoo et al. 1997), when EGFR in the STB appears to be activated primarily by other ligands in an autocrine manner (Ladines-Llave et al. 1991; Lysiak et al. 1993; Lysiak et al. 1995; Tanimura et al. 2004). At term, EGFR protein is abundant on the microvillus and basolateral surfaces of STBs (Rao et al. 1985), suggesting activation by maternal and fetal ligands, respectively. It is also detected in intracellular organelles, including the endoplasmic reticulum, Golgi apparatus, and lysosomes, consistent with ongoing synthesis and degradation (Ramani et al. 1986). The complex spatiotemporal distribution of EGFR and its ligands strongly suggests the necessity for precise regulation of the receptor over the course of pregnancy.

One additional signaling molecule worth mentioning, although not formally recognized as an EGFR ligand, is EGF-like domain 7. EGFL7 is a secreted angiogenic factor whose overexpression has been positively correlated with the phospho-activation of EGFR. Conversely, knockdown of the *EGFL7* gene was shown to inhibit activation of EGFR signaling cascades, including the PI3K/Akt and MEK/ERK pathways (Luo et al. 2014; Wang et al. 2017; Liu et al. 2018). Generally speaking, adult tissues express little to no *EGFL7* except in those tissues that are heavily vascularized such as lung, heart, kidney, ovary, and uterus. EGFL7 mRNA is abundantly expressed in both human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC), whereas a variety of nonendothelial cell lines have been found to express little to none (Fitch et al. 2004). Under normal conditions, EGFL7 promotes VEGF-induced sprouting angiogenesis and supports endothelial barrier function via tyrosine phosphorylation of VE-cadherin, while knockdown of *EGFL7* inhibits these processes (Usuba et al. 2019). Primary human trophoblasts have

been shown to respond to hypoxia by increasing *EGFL7* transcription, presumably in an attempt to improve cellular oxygenation. When cultured in 1% O2 for 24 hours, the cells exhibited a HIF1α-independent 2.2-fold upregulation of EGFL7 mRNA ($P < 0.0001$) (Whitehead et al. 2018).

EGFR in fertility and fetal development

Egfr knockout studies in mice have revealed the vital function of EGFR in pregnancy, as evidenced by the fact that embryos of whole body *Egfr* knockout mice die in gestation (Sibilia & Wagner 1995; Threadgill et al. 1995). To determine the importance of EGFR and other members of the ERBB family of receptors in fertility, Large et al. (2014) created a mouse model in which *Egfr* expression was ablated exclusively in the uterus, leaving expression at normal levels in all other tissues. Of the seven females tested, four gave birth to only a few pups in a six-month period, and three produced no pups at all. This appeared to be related to incomplete decidualization caused by the absence of expression of critical EGFR effectors such as Bmp2 (Lee et al. 2007) and Wnt4 (Franco et al. 2011), which mediate decidual cell proliferation, survival, and differentiation. In comparison, knockout of uterine *Erbb2 (Her2)* or *Erbb3*, which are also expressed in the endometrium, produced only a small reduction in average litter size, suggesting that EGFR is the only member of the ERBB receptor family that is necessary and sufficient for proper endometrial function. Large *et al.* further demonstrated the essential role of EGFR in decidualization using a primary human endometrial stromal cell (HESC) culture model. Decidualization markers such as IGFBP1 and prolactin are known to be induced in HESC culture when these cells are treated with a combination of estrogen, progestin and cAMP (Giudice et al. 1992; Huang et al. 1987). However, expression of these markers was significantly reduced when HESC was transfected with EGFR siRNA, and the cells exhibited an aberrant morphology (Large et al. 2014).

EGFR is also involved in preimplantation development of the embryo. Administration of EGF has been shown to enhance the *in vitro* rate of blastocyst formation of several species, including mice (Dadi et al. 2007), pigs (Abeydeera et al. 1998; Wei et al. 2001), cows (Lonergan et al. 1996; Watson et al. 2000), and sheep (Guler et al. 2000). In cultures of fertilized sheep oocytes, for example, EGF more than doubled the number of blastocysts formed (1.4+/−0.1 vs. 0.6+/−0.2 per ewe) for the duration of the culture period (Grazul-Bilska et al. 2003). The most important EGFR ligand in the earliest days of pregnancy, however, may be HBEGF. The functions of HBEGF are numerous, including a central role in the establishment of pregnancy, development of human embryos to the blastocyst stage and beyond, and mediation of trophoblast motility (reviewed in Jessmon et al. 2009). During implantation, endometrial HBEGF serves as both a pro-survival growth factor and, in its transmembrane "proHBEGF" form, an attachment factor for blastocysts. Plasma membrane-tethered proHBEGF binds to either EGFR or heparin sulfate proteoglycan on the surface of an adjacent blastocyst, facilitating both the adherence of the blastocyst and the initiation of juxtacrine signaling (Raab et al. 1996).

EGFR promotes trophoblast proliferation

EGFR ligands have been shown to promote trophoblast proliferation in vitro. In cultures of first trimester human trophoblasts, EGF (Maruo et al, 1992), TGFα (Lysiak et al. 1993), and AREG (Lysiak et al. 1995) were each shown to increase the rate of proliferation. Filla et al. (1993) additionally showed that EGF and TGFα stimulated growth in cultures derived from second trimester trophoblasts. The unique expression profiles of EGFR and HER2 in first trimester CTB further suggest that EGFR mediates proliferation, as evidenced by the presence of EGFR and absence of HER2 in proliferative villous CTB. Inversely, HER2 appears to mediate invasion and differentiation, since HER2 was expressed in invasive, extravillous CTB, but EGFR was undetected in these non-proliferative cells (Jokhi et al. 1994). However, in cultured CTB derived from term placentas, EGFR activation by EGF induced differentiation into STBs (Morrish et al. 1987), suggesting that the role of EGFR changes over the duration of pregnancy.

EGFR signaling protects trophoblasts from apoptosis

Apoptosis, or programmed cell death, is a necessary occurrence in the differentiation of tissues. The hypoxic environment and elevated levels of inflammatory cytokines observed in PE placentas, however, can stimulate excessive apoptosis, resulting in placental dysfunction and retarded growth. One of the most important functions of EGFR during pregnancy is protection against apoptosis. Indeed, EGFR activation can protect against apoptosis induced by hypoxia (Perkins et al. 2002; Armant et al. 2006; Humphrey et al. 2008), reactive oxygen species (Moll et al. 2007), and inflammatory cytokines (Garcia-Lloret et al. 1996) in a number of cell models. Interestingly, supraphysiological concentrations of EGF (>50 ng/ml) provided reduced protection against cytokine-induced trophoblast death, suggesting that *excessive* stimulation of EGFR activity can also harm trophoblast viability (Garcia-Lloret et al. 1996), perhaps due to compensatory downregulation of the receptor itself, or associated signaling kinases.

EGFR signaling and angiogenesis

While under normal circumstances VEGF is the key regulator of angiogenesis in the vascular endothelium, treatment of HUVEC with HBEGF or EGF can induce VEGF-independent migration, capillary tube formation, and angiogenesis (Mehta & Besner 2007). HBEGF also strongly stimulated expression of eNOS mRNA, increased eNOS protein synthesis, and boosted the release of nitric oxide (NO). Pretreatment of HUVEC with the PI3K kinase inhibitor wortmannin or the EGFR kinase inhibitor AG1478 blocked these effects, showing EGFR-mediated eNOS activity to be regulated via the PI3K pathway (Mehta et al. 2008). EGFR expression in endothelial cells tends to be modest, as stated earlier, but these data suggest that EGFR could assume direct control of angiogenesis and enhance eNOS function in an environment where it outcompeted VEGFR1/2 for access to the relevant effector molecules.

Soluble EGFR in the placenta

In addition to its robust expression of mRNA encoding the full-length 170 kDa EGFR, the placenta also expresses a number of naturally occurring alternative *EGFR* transcripts

that encode truncated variants of the full-length receptor termed "soluble EGFR" (sEGFR). These include the 1.8-kb mRNA transcript that encodes a secreted 60 kDa isoform (p60 EGFR) and the 3.0-kb transcript that encodes a plasma membrane-tethered 110 kDa isoform (p110 EGFR) (Reiter & Maihle 2003). Both sEGFR variants lack all of the typical domains of the full-length receptor except the extracellular ligand-binding domain, rendering them devoid of any kinase activity. These alternative transcripts are poorly expressed in most human tissues, but the highest expression level occurs in placenta (Reiter & Maihle 2003).

Since the ligand-binding domain of sEGFR isoforms is identical to that of the full-length receptor, sEGFR isoforms are capable of forming complexes with EGFR ligands, but does not induce sEGFR homodimerization (Gunther et al. 1990; Flickinger et al. 1992). sEGFR is generally thought to be a negative regulator of EGFR signaling, but it is unclear whether inhibition of EGFR kinase activity is achieved by "mopping up" available ligand (Flickinger et al. 1992) or by dimerizing with the full-length EGFR (Basu et al. 1989). In hindsight, it seems likely that these two distinct actions—ligand binding and dimerization with full-length EGFR—are distinct steps in a single mechanism.

Dysregulation of EGFR during pregnancy

EGFR in preeclampsia

Relatively few studies have addressed the subject of EGFR signaling in the context of PE. A transcriptome meta-analysis found that the EGFR gene and transcription regulators of EGF-dependent pathways were significantly upregulated in preeclamptic placentas, leading the authors to postulate that EGFR was among the 50 or so genes "most relevant to preeclampsia" (Moslehi et al. 2013). Surprisingly, the upregulation of EGFR and its transcriptional regulators was associated with a deficiency of EGFR signaling rather than an increase (Moslehi et al. 2013), which is supported by observations of stunted syncytial differentiation in PE (reviewed in Huppertz 2011). When cultured trophoblasts were exposed to a hypoxic environment for the first 24 hours, they exhibited significantly more apoptosis than cells experiencing hypoxia at 48 to 72 hours, suggesting that less differentiated trophoblasts are more vulnerable to the negative effects of hypoxia than are more differentiated trophoblasts (Levy *et al.* 2000). This is also consistent with the characterization of PE as a condition of reduced EGF/EGFR signaling (Moslehi et al. 2013), since EGF is known to promote trophoblastic differentiation (Morrish et al. 1987). In contrast, Hastie et al. (2019) found evidence that EGFR mRNA expression and phosphorylation of EGFR and ERK1/2 were significantly increased in severe early-onset preeclamptic placentas compared with control placentas, resulting in the secondary upregulation of sFlt1. The authors speculated that the increased EGFR pathway activity in preeclamptic placenta might be a response to hypoxia. EGFR mRNA and protein expression were found to be elevated in primary cytotrophoblasts cultured in 1% oxygen for 24 h, just as in severe early-onset PE. Polymorphisms of EGF (Chenthuran et al. 2014) and HBEGF (Harendra et al. 2012) have also been associated with PE, further suggesting that aberrant EGFR signaling can have negative consequences during pregnancy.

Too little or too much EGFR activity can be harmful during pregnancy

Since, as previously noted, the complete absence of EGFR results in embryonic lethality, it is appropriate to investigate the activity level of EGFR required for healthy placental development. To study the physiological consequences of *attenuated* EGFR signaling, Dackor et al. (2009a) created strains of mice that were homozygous for a *hypo*morphic allele of *Egfr* (termed '*Egfr*^wa2'). The spongiotrophoblast layer of the placentas of mice expressing *Egfr*^wa2 was significantly thinner or absent, depending on the strain. Embryo growth was retarded in the third week of development, leading the authors to suggest that *Egfr*^wa2 homozygous mice could model intrauterine growth restriction (IUGR) in humans. Additionally, to study the effects of *excessive* EGFR signaling, Dackor et al. (2009b) created strains of mice that were homozygous or heterozygous for the *hyper*morphic *Egfr*^Dsk5 allele. *Egfr*^Dsk5 heterozygous females exhibited enlarged placentas with a thickened spongiotrophoblast layer, which was associated with low fertility and pregnancy loss in some strains, independent of embryo genotype. *Egfr*^Dsk5 homozygotes showed even greater placental enlargement, and this too was often associated with embryo death prior to day 15.5. Together, these experiments support the idea of a "Goldilocks zone" for maternal and fetal EGFR signaling, in which EGFR plays an essential role in pregnancy, but either too little or too much activity can have deleterious consequences. As previously mentioned, PE has already been associated with reduced EGFR signaling. Is it possible that *excessive* EGFR activity could also, at least in some cases, exacerbate the symptoms of PE?

EGFR ligand expression in PE

The current evidence for expression levels of EGFR and its ligands in PE is limited and contradictory. Kosovic et al. (2017) compared EGF and EGFR expression separately in decidual cells (DC), villous trophoblasts (VTB), and extravillous trophoblasts (EVTB) obtained from either normal (38 wks) or preeclamptic placentas (38 wks). There was no significant difference between the normal and PE groups in maternal and gestational age, neonatal sex, or birth weight. No between-group differences were found in the expression levels of EGF or EGFR in DC, VTB, or EVTB. Similarly, no difference was observed in urine EGF concentrations at 10-18 weeks between mothers with PE and healthy matched controls (Lindqvist et al. 1999). Alternatively, immunohistochemical labeling of six EGFR ligands (EGF, HBEGF, TGFα, AREG, EREG, and BTC) in the trophoblasts of placentas from women with PE (mean 32.6 wks), preterm labor (32.9 wks), small for gestational age fetuses (32.9 wks), and normal term placentas (39 wks) found that EGF, HBEGF, and TGFα were significantly reduced in the basal plate of PE placentas compared to all other groups (Armant et al. 2015). One study of *serum* EGF concentration in PE pregnancy (32 wks, median = 93.13 pg/ml) found EGF levels to be more than double those observed in normotensive pregnancy (38.5 wks, median = 38.82 pg/ml) (Kupsamy et al. 2019). Yet another study reported that when maternal serum levels of EGF, HBEGF, and TGFα were measured in 20 pregnant women of similar maternal and gestational age (~34 wks) with or without PE that eventually delivered at term, the median serum concentration of EGF in the PE group (147.5 pg/ml) was less than half that of the median level in the normotensive group (324.3 pg/ml). Interestingly, however, levels of HBEGF and TGFα were not reduced (Armant et al. 2015), and other evidence suggests HBEGF may actually be significantly elevated (Charkiewicz et al. 2018). This is physiologically significant if true, because

HBEGF appears to be the most commonly utilized ligand in known ligand-dependent EGFR transactivation mechanisms.

To summarize, these confusing data on EGFR ligand expression in PE illustrate why the expression of EGFR ligands in PE and their potential role in the etiology and maintenance of the disease is far from settled. It is likely that each subtype of PE expresses unique tissue-specific levels of EGF ligands, but the impact of these differences on PE pathology remains unclear.

Soluble EGFR in PE

One novel way that the availability of EGFR ligands could be increased in some types of PE is by reduced expression of sEGFR. Measurement of serum concentrations of sEGFR in 172 normotensive or preeclamptic pregnant women showed levels to be significantly lower in preterm PE (33.0 ± 2.68 wks, $P < 0.001$) and term PE (38.8 ± 1.14 wks, $P < 0.01$) than matched controls. Conversely, sFlt1 was significantly *higher* in preterm preeclampsia ($P < 0.001$) and term preeclampsia ($P < 0.01$) than matched controls, and the sFlt1:sEGFR ratio correlated strongly ($R = 0.711$) with the severity of preterm preeclampsia (Cui et al. 2018a). Although serum sEGFR level alone could not differentiate between mild and severe forms of preterm PE, alterations in the levels of sEGFR and three other proteins (VEGF ↑, sEndoglin ↑, PlGF ↓) were each shown to be independent predictive markers for PE, (Cui et al. 2018b). Citing known effects of EGFR on vascular tone and sodium retention in arterial hypertension (Beltowski & Lowicka 2009), Cui *et al.* speculated that reduced expression of serum sEGFR in PE might increase the availability of EGFR ligands in the vasculature, thereby boosting EGFR signaling and exacerbating hypertension in PE. Ilekis et al. (1995) found an inverse relationship in the placenta between expression of the p60 sEGFR transcript and that of the full-length EGFR transcript, opening the possibility that a high concentration of the full-length EGFR protein may necessitate a low concentration of sEGFR and vice versa. This means that higher expression of EGFR may typically be accompanied by greater availability of ligands, further potentiating EGFR activity.

EGFL7 in PE

Initial measurements of changes in EGFL7 expression in PE pregnancies have been contradictory. Whitehead et al. (2018) observed no significant difference in EGFL7 mRNA expression in early or late onset PE compared to gestational age-matched normotensive controls. However, elevations in EGFL7 protein levels of PE patients were reported by Massimiani et al. (2019), who were unable to detect circulating EGFL7 in nonpregnant women but found that it was detectable during pregnancy. In PE patients, the mean circulating level of EGFL7 protein at weeks 35-40 was significantly higher (44 vs. 25 μg/mL). Similarly, villous explant cultures from PE placentas at weeks 35-37 released three times as much EGFL7 into the culture media in 24 hrs (mean = 257.99 μg/mL) than did explant samples from controls at weeks 37-39 (mean = 86.76 μg/mL). Notably, the variance was high between samples, with many PE-derived samples exhibiting no difference from controls and others showing stark differences, suggesting that elevated EGFL7 may only be a feature of particular PE subtypes. Thus, while EGFL7 may play a role in the response to hypoxia, it is not likely to be a fundamental initiating event. In the absence of EGFR

elevation, we doubt changes in EGFL7 would be a principal determinant of endothelial dysfunction. However, in endothelial cells with elevated EGFR expression, EGFL7 might further drive excessive EGFR activity.

EGFR transactivation

One last important EGFR activation mechanism that may yet be found relevant in PE is "transactivation" by agonists of G-protein coupled receptors (GPCRs) via induction of matrix metalloproteinase (MMP) cleavage of membrane-tethered EGFR ligand (e.g. proHBEGF, proEGF, etc.). GPCR-mediated EGFR transactivation can also occur independently of MMP activity or ligand through interaction with a specific Src family kinase (SFK), such as in the case of the muscarinic M2 acetylcholine receptor in COS-7 cells via a mechanism that involves direct association of EGFR with Fyn, but is independent of Src and Yes (Stirnweiss et al. 2006). Cytokines such as TNFα, IL-8, IL-1α, IL-1β, and IFNγ are known to be significantly elevated in PE (reviewed in LaMarca et al. 2007; Lau et al. 2013, Bird et al. 2013), and all of these have been reported to transactivate EGFR (Argast et al. 2004; Chen et al. 2004; Itoh et al. 2005; Lee et al. 2015; Tanida et al. 2004). IL-8 transactivation of EGFR, for example, has been observed in microvascular endothelium (Schraufstatter et al. 2003), and it has been shown to be capable of inducing phosphorylations in Src, ERK1/2, FAK, Jnk, p38-MAPK, STAT1 and STAT3 (Kyriakakis et al. 2011). Interestingly, in addition to transporting EGFR, placental exosomes may also be a source of these transactivating cytokines. When BeWo cells, a placental choriocarcinoma cell line commonly used as an *in vitro* model for the placenta, were treated with exosomes isolated from the serum of early onset PE or late onset PE pregnancies, expression of the *pro*-inflammatory interleukin IL-8 increased, and expression of the *anti*-inflammatory interleukin IL-10 decreased (Maduray et al. 2020).

A new model for endothelial dysfunction in preeclampsia

Pregnancy-adapted programming of the endothelium

Pregnancy requires significant enhancements of vascular function to meet the needs of the mother and developing fetus, including increased cardiac output, blood volume, and capacity for vasodilation. These adaptations are dependent on numerous changes in cell signaling that we have termed 'pregnancy-adapted programming.' One particular adaptation we have studied extensively is the augmented capacity for Cx43 gap junctional communication. Using cultured uterine artery endothelial cells obtained from pregnant ewes (P-UAEC), which retain their pregnancy-enhanced capacity for pro-vasodilatory signaling (Bird et al. 2000; Gifford et al. 2003), we have shown Cx43 gap junctional enhancement to be necessary and sufficient for optimizing the intercellular Ca2+ signaling that drives sustained pregnancy-enhanced NO production (Yi et al. 2010). A consequence of this fact is that any signaling event that diminishes Cx43 gap junctional communication could potentially inhibit pregnancy-adapted vasodilation. Supporting this assertion, we have established that treatment of P-UAEC monolayers with growth factors and cytokines reduces the agonist-induced Ca2+ bursting otherwise required for pregnancy-adapted vasodilation [Figure 1] (Yi et al. 2011; Boeldt et al. 2015; Ampey et al. 2019).

Recently, we have shown more specifically that Ca2+ bursting is attenuated in P-UAEC via Src- and ERK-mediated phosphorylations of Cx43 in gap junctional plaques (Boeldt et al. 2015, Ampey et al. 2019). Since these same phosphorylation events occur in wound healing, it is worthwhile to review briefly the local environment of an acute wound site. When tissue is wounded, the immediate elevation of growth factors and cytokines results in inhibitory phosphorylations of Cx43 (reviewed in Barrientos et al. 2008; Widgerow 2012). The inhibition of Cx43 function reduces cell-to-cell connectivity, which allows the removal of damaged tissue and the proliferation and migration of new cells into the wounded area. As this process completes, the local concentrations of growth factors and cytokines return to basal levels and Cx43 function returns to normal (Coutinho et al. 2003). In a chronically wounded state, however, the wound site may continue to exhibit elevated concentrations of cytokines, including TNFα, IL-1, and IL-6, and growth factors such as VEGF, bFGF and EGF (reviewed in Barrientos et al. 2008), resulting in sustained inhibition of Cx43 gap junctional assembly and channel function (Laird 2005; Moreno 2005). Indeed, the parallels between wound healing and the suppression of vascular adaptation to pregnancy have led us to ask if the poor vasodilation observed in PE is in fact due to the same signaling pathways observed in chronic wounding (reviewed in Bird et al. 2013). Could some subclasses of PE essentially be the body mistakenly reacting to pregnancy as though it were a non-healing wound?

EGFR-mediated endothelial dysfunction in cultured uterine artery cells

We have recently confirmed that one such pregnancy-associated and wounding site growth factor, VEGF (acting through VEGFR2), induces inhibitory phosphorylations of Cx43 in P-UAEC via Src and ERK (Boeldt et al. 2015). EGF has also been shown to inhibit gap junctional communication (GJC) in several cell types (Madhukar et al. 1989; Lau et al. 1992; Oh et al. 1993); however, EGF is not able to inhibit Ca2+ bursting in P-UAEC as VEGF does, suggesting that the low endogenous receptor number (~7000 EGFR/cell) is insufficient to couple to the signaling pathways that mediate inhibitory Cx43 phosphorylations (Clemente et al. 2020). To determine if the EGF/EGFR system could substitute for the VEGF/VEGFR2 system, we overexpressed EGFR in P-UAEC via adenoviral transduction. Although these P-UAEC now exhibited constitutive EGFR autophosphorylation, this high basal activity was not sufficient to suppress Ca2+ bursting. However, adding EGF treatment to these modified cells induced phosphorylations of ERK1/2 and Cx43, and inhibited Ca2+ bursting to the same degree as VEGF. Interestingly, VEGF treatment no longer inhibited Ca2+ bursting in P-UAEC with added EGFR, as if EGFR had 'hijacked' the molecular machinery formerly utilized by VEGFR2 [Figure 2]. We tentatively proposed that overexpression of EGFR resulted in receptor localization in membrane regions where it was normally absent and so could now recruit pools of key effector molecules (e.g. adapter proteins, intermediate kinases, etc.) away from VEGFR2, and allowing EGFR to mediate Cx43 phosphorylation instead. If this is indeed the case, it follows that the low endogenous EGFR expression in P-UAEC in vivo may be necessary for VEGFR-2 to retain control of ERK-mediated regulation of GJC, a committing step to initiating angiogenesis (Clemente et al. 2020).

The observation that EGFR could induce PE-like endothelial cell dysfunction in our experimental model led us to ask if this mechanism might actually be active in the uterine arteries of women with PE. For our *in vitro* model of EGFR-mediated dysfunction to occur *in vivo*, the uterine arteries would need to overexpress EGFR; however, excess EGFR (and perhaps its ligands) might not need to be synthesized by the arterial endothelium. It could instead come from the richest source of EGFR in the mother's body - the placenta - through the shedding of EGFR rich exosomes and other extracellular vesicles.

Could placenta-derived EGFR suppress pregnancy-adapted vasodilation in PE?

Exosomes are extracellular vesicles that originate in the endocytic pathway, characterized by a cup-shaped appearance and a diameter of 40 to 160 nm (reviewed in Kalluri & LeBleu 2020). They are secreted by many tissue types and possess common identifying markers (e.g. CD63). Exosomes of placental origin are uniquely identified by the presence of placental alkaline phosphatase (PLAP), an enzyme isoform produced exclusively by the placenta (Sabapatha et al. 2006). Placental (PLAP+) and non-placental exosomes can be detected in maternal plasma at six weeks of pregnancy and increase in concentration throughout the first trimester (Salomon et al. 2014; Sarker et al. 2014). In healthy pregnant women, plasma concentration of exosomes in the first trimester (N = 20, 6-12 weeks) was found to be 50X higher than measured in non-pregnant women (N = 9), and the level of PLAP+ exosomes increased markedly in each subsequent trimester (Salomon et al. 2014). In preeclamptic pregnancies, Pillay et al. (2016) found that the plasma concentration of PLAP+ exosomes increased in the third trimester only in early onset PE and decreased in plasma from late onset PE, suggesting that the shedding of PLAP+ exosomes may be dependent on PE type. Furthermore, extracellular vesicles (EVs) from healthy placentas and diseased placentas differ in content. When peripheral blood mononuclear cells were exposed to microparticles from the plasma membranes of hypoxic trophoblasts, they experienced a faster and more robust inflammatory response than when the cells were exposed to microparticles from normal trophoblasts (Lee et al. 2012). Similarly, microparticles from the peripheral venous blood of healthy pregnant women protected against apoptosis and promoted migration and tube formation in early-stage trophoblasts. In contrast, these processes were inhibited when the cells were incubated with microparticles from women with gestational hypertension or PE (Shomer et al. 2013). Another study found that the content, quantity, and bioactivity of CTB-derived exosomes obtained from conditioned media correlated inversely with oxygen tension. Exosomes from the most hypoxic environment (1% O_2) stimulated the greatest invasion and proliferation of EVTB, suggesting that exosome-induced EVTB migration is a normal adaptive response to placental hypoxia. The authors concluded that CTBs regulate EVTB phenotype by altering the protein content and secretion rate of exosomes in response to oxygen tension (Salomon et al. 2013). In this context, Whigham et al. (2019) proposed using the content of placenta-derived EVs in the maternal circulation as a tool for diagnosing PE and other diseases of pregnancy, and designing appropriate therapies.

Placental trophoblasts are known to express increasing amounts of EGFR mRNA and protein over the course of pregnancy (Chen et al. 1988; Magid et al. 1985), and we have identified shed PLAP+/EGFR+ extracellular vesicles in first trimester plasma samples using

nanoscale high resolution flow cytometry (Clemente et al. 2020). These observations led us to hypothesize that some of these placenta-derived EGFR+ exosomes and/or microparticles could be taken up by the arterial endothelium, thereby transferring EGFR from the placenta to the uterine and systemic vasculature. While exosomal transfer of RNAs, proteins, etc. appears to be a normal part of pregnancy, excessive transfer of EGFR, its ligands, and associated signaling molecules in endothelial cells could negatively impact maternal vasodilation and perhaps drive some subtypes of PE [Figure 3].

Although this mechanism has yet to be demonstrated in PE, cellular pathology due to transfer of EGFR+ exosomes has been observed in cancer. Gastric cancer cells have been shown to secrete EGFR+ exosomes that fuse with the plasma membranes of liver stromal cells. The exogenous EGFR facilitates cancer proliferation by providing an otherwise absent binding site for the c-MET receptor on metastatic cancer cells (Zhang et al. 2017). Similarly, exosomes secreted by cultured hypoxic prostate cancer cells have been reported to induce prostate stromal cells to exhibit a cancer-associated fibroblast phenotype and become more invasive (Ramteke et al. 2015). More recently, transfer of exosomal wild-type EGFR to cancer cells that express activating EGFR mutations was found to induce resistance to the tyrosine kinase inhibitor osimertinib (Wu et al. 2021). It should also be noted that although we are the first to specifically propose EGFR itself from placental vesicles as a driver of endothelial cell dysfunction in PE, negative effects of placental vesicles on vasodilation were first reported over two decades ago. When STB microvillous membrane vesicles were perfused through preconstricted subcutaneous arteries obtained from fat biopsies, the arteries displayed a reduced relaxation response to acetylcholine treatment (Cockell et al. 1997). Additionally, overnight incubation with microparticles from preeclamptic serum caused abolishment of the bradykinin-mediated relaxation otherwise observed in the presence of healthy pregnant microparticles (van Wijk et al. 2002).

When considering the plausibility of placental EGFR-mediated endothelial dysfunction as a factor in PE, it is sensible to ask why no one has reported observing this mechanism before. However, two key features of the mechanism explain why it might have eluded detection. Firstly, since the putative influx of EGFR would originate in the placenta, *transcriptome* analysis of endothelial cells from PE patients would not detect it. Secondly, cultured endothelial cells (e.g. HUVEC) from PE patients would quickly lose any exogenous EGFR due to cell division and protein turnover with passage in 'normal' serum conditions. Therefore, only the direct quantification of PLAP and EGFR proteins in *freshly isolated* endothelium from PE patients could reveal the existence of such a mechanism. Leavey et al. (2015) used the term "canonical" to name the early onset subtype of PE characterized by upregulation of known PE markers such as sFlt1 and sENG, which are not significantly elevated in the other subtypes. The authors described this well-defined cluster as a disorder in which the placenta appears as "an injured tissue in a hypoxic environment, secreting increased amounts of placental products into the maternal compartment." If placental hypoxia does indeed stimulate greater EGFR expression and activity that results in increased sFlt1 levels (Hastie et al. 2019), then our proposed mechanism of EGFR-mediated endothelial dysfunction is most likely to be detected in cases of early onset PE that exhibit elevated sFlt1.

Conclusions

The complex spaciotemporal regulation of expression of EGFR and its ligands in reproductive tissues over the course of pregnancy suggests distinct developmental roles for the receptor. EGFR is critical for both fertility and fetal development. In the placenta, it promotes trophoblast proliferation and protects trophoblasts from apoptosis. Insufficient EGFR activity is associated with pathologies of pregnancy such as IUGR and PE, and excessive EGFR activity is known to produce aberrant placental development in at least one experimental model, but the effects of EGFR overexpression *in the endothelium* remain unclear. The findings outlined above suggest that it could indeed impair normal angiogenesis, allow EGF to impair otherwise pregnancy-adapted vasodilation of the maternal vasculature, and provide novel transactivation pathways for other endocrine factors.

Based on the steadily increasing rate of placental (PLAP+) exosome and microparticle secretion into the maternal vasculature with each successive trimester, and our recent observation of PLAP+/EGFR+ extracellular vesicles (EVs) in maternal plasma, it appears likely that some level of EGFR transfer from the placenta to the vascular endothelium is a normal part of pregnancy. Under ordinary circumstances, this is likely benign, or even beneficial, perhaps increasing placental EGFR transfer with time to increasingly limit VEGFR2 function and slow angiogenesis in the second half of pregnancy. EVs from healthy placentas have even been shown to induce positive adaptive responses when administered to endothelial cells in hypoxic conditions, appearing to attempt restoration of healthy cellular oxygenation. Given exosomal effects appear to be a double-edged sword, we should also acknowledge there may also be dependence on other cargo in the exosomes (e.g. signaling adaptors, kinases, miRNA) that are altered in the hypoxic and/or PE placenta. It is also important to note this proposed mechanism is not unique to pregnancy. Our recent findings that EGFR overexpression in cultured P-UAEC remaps signaling pathways away from VEGFR2 and artificially suppresses the Cx43-dependent Ca2+ signaling required for sustained vasodilation are similar to a mechanism reported *in vivo* in cancer cells, whereby EVs secreted by tumors promote proliferation and metastasis by incorporating into healthy cells and hijacking normal processes using newly delivered EGFR. We speculate that increased vesicle shedding from the placenta over time in response to hypoxia above that of normal pregnancy may mediate symptoms of hypertensive PE by transferring *too much* EGFR protein (and possibly EGFR ligands and signaling complexes) to the endothelium. This mechanism is also consistent with the observation that the only truly effective treatment for PE to date is removal of the placenta.

Acknowledgments

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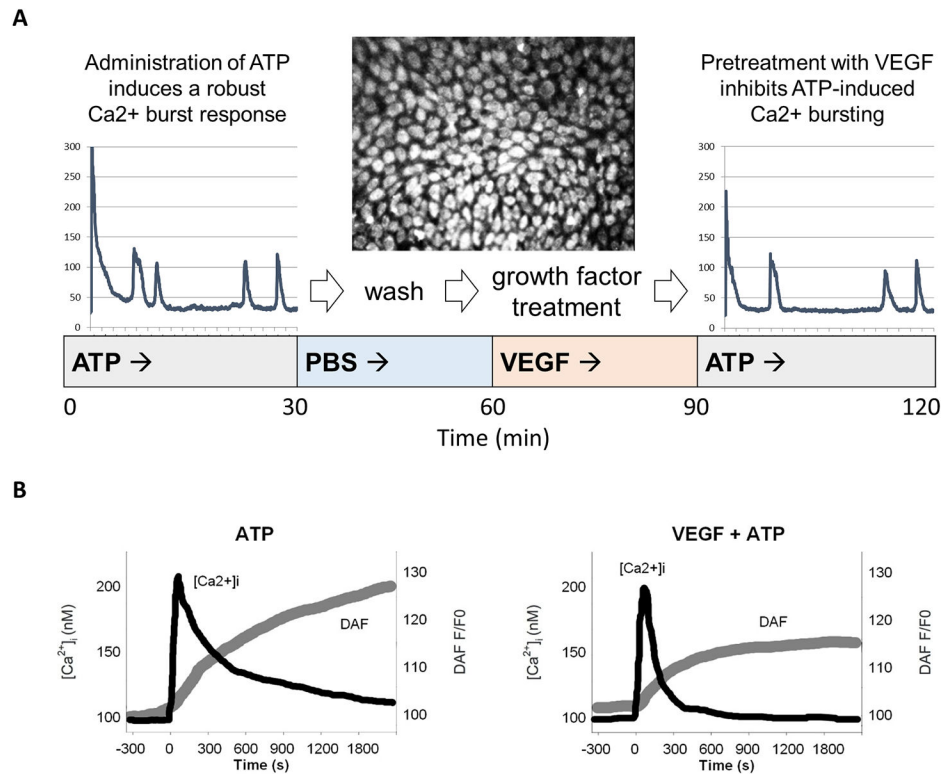
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A

Administration of ATP induces a robust Ca2+ burst response

wash → growth factor treatment →

Pretreatment with VEGF inhibits ATP-induced Ca2+ bursting

| ATP → | PBS → | VEGF → | ATP → |

Time (min)

B

Figure 1. VEGF pretreatment of an endothelial cell monolayer inhibits the ATP-induced Ca2+ burst response.

Pooled passage 4 P-UAEC grown in 35-mm glass-bottom microwell dishes to 95-100% confluence were then loaded with Fura-2 AM, a free Ca2+ dye. Cells were then incubated with 100 μM ATP and the data recorded for 80-100 cells simultaneously for 30 min. After the initial ATP treatment, the dish was washed and allowed to sit for 30 min. Then, VEGF (or another growth factor or cytokine) was added and recorded for 30 min before a subsequent treatment of ATP was added for an additional 30 min. Ca2+ bursts were then counted for the "before" and "after" VEGF treatments. Finally, before and after counts of cell burst numbers were compared (Boeldt et al. 2015). (A) The tracing from a single cell stimulated with AP before (left) or after (right) treatment with VEGF is shown. Note the narrowing of the initial peak and the fewer subsequent peaks of Ca2+ following VEGF treatment, especially in the period 5 to 15 minutes. These findings are consistent with (B), where intact human umbilical vein tissue is loaded with Fura-2 and DAF-2 (to measure free Ca2+ and NO, respectively) and treated with ATP before and after VEGF treatment (Yi et al. 2010). This data shows overall endothelium response, i.e. the mean level of Ca2+ elevated above the baseline and NO production. Before VEGF exposure, Ca2+ and NO responses are robust. Following VEGF treatment, the mean level of free Ca2+ falls more quickly, and NO production is suppressed.
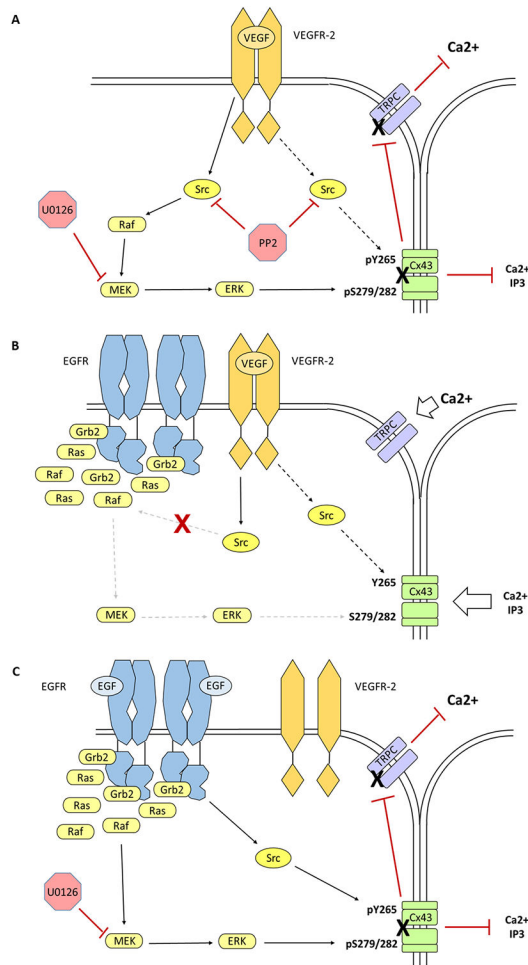
Figure 2. Overexpression of EGFR in P-UAEC suppresses the ability of VEGFR2 to activate Cx43-associated ERK1/2.

(A) VEGF administration (10 ng/mL) triggers the formation of activated VEGFR-2 homodimers, which initiates Src-mediated phosphorylation of Cx43 Y265 and Src-dependent ERK-mediated phosphorylation of Cx43 S279/282, which results in gap junctional gate closure and loss of periodic TRP channel-mediated Ca2+ entry needed for pregnancy-adapted vasodilation. While the exact nature of communication between Cx43 opening and TRPC channel activation remains unclear, pharmacological inhibition of either Src (PP2, 10 μM) or MEK (U0126, 10 μM) protects the sustained Ca2+ burst response from VEGF-induced downregulation. (B) Overexpression of EGFR appears to have significant effects on the local environment at the plasma membrane, as evidenced by the fact that VEGF was not able to inhibit ATP-stimulated Ca2+ bursting in the presence of concentrated EGFR. We proposed that the overexpression of EGFR in P-UAEC localizes the receptor in membrane regions where it is normally absent, thereby conferring access to effector molecules that it would otherwise be denied. The constitutively autophosphorylated C-terminal tail of EGFR serves as a continuous source of active phosphotyrosine docking sites, thereby acting as a sink for effector molecules such as SOS, Grb2, the Ras-GTPase, Raf-1, Shc, Cbl and PLCγ. This effectively reduces their availability for other receptors such as VEGFR2. Of note, EGFR does not prevent VEGF-activated Src phosphorylation of Cx43

Y265, which is known to initiate gap junction disassembly but is apparently not associated with acute gate closure of the gap junction. (C) Normally, treatment of P-UAEC with EGF has no significant effect on gap junctional communication or Ca2+ bursting, presumably due to the low expression level of EGFR. However, when P-UAEC that *overexpress* EGFR are treated with EGF (10 ng/mL), Src- and ERK-mediated phosphorylations of Cx43 occur and Ca2+ bursting is inhibited. Unlike VEGFR2-mediated Ca2+ burst inhibition, the activation of MAPK pathway by EGFR does not require Src. Only pretreatment with the MEK inhibitor U0126 protects against EGFR-induced gap junctional closure and impaired Ca2+ bursting.
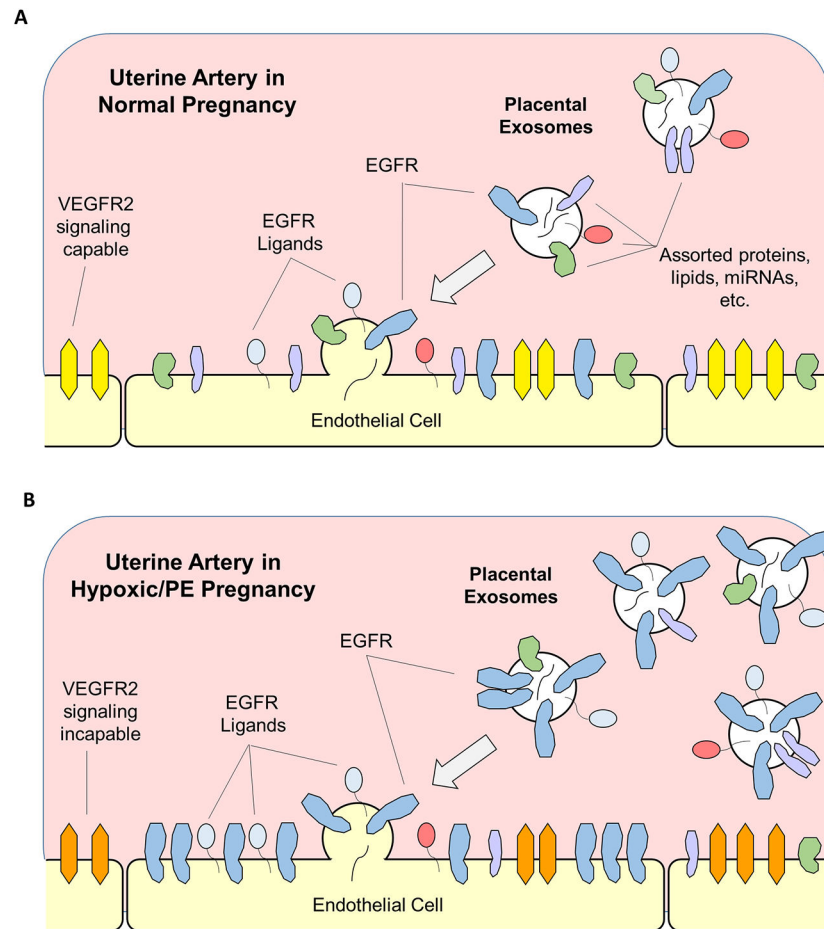
Figure 3. Putative mechanism for EGFR-mediated endothelial cell dysfunction in PE.
(A) Exosomes and microparticles containing a variety of proteins, micro RNAs, etc. are released from the placenta into the uterine and systemic vasculature, some of which may fuse with arterial endothelium. This exosomal fusion is likely to be benign or beneficial. As delivery of exogenous EGFR (and potentially ligands, EGFL7, etc.) increases with time, there may be a limit placed on the ability of VEGFR (yellow) to control endothelial function and particularly late term angiogenesis. (B) In PE pregnancy, where exosomal delivery may further increase, excessive delivery of EGFR and related signaling molecules to the endothelial plasma membrane now overwhelms the VEGFR2 (orange) signaling system completely, rendering the receptor incapable of endothelial cell control. The hypoxia and/or inflammation associated with PE is likely to alter not only EGFR concentration, but signaling adaptors, kinase subtypes, and miRNA co-delivered in the same PE placenta-derived vesicles, reflecting the placenta response to physiologic distress.