Published in final edited form as:

Mol Cell Endocrinol. 2020 January 01; 499: 110590. doi:10.1016/j.mce.2019.110590.

Adenoviral Transduction of EGFR into Pregnancy-Adapted Uterine Artery Endothelial Cells Remaps Growth Factor Induction of Endothelial Dysfunction

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Abstract

During pregnancy, uterine vascular vasodilation is enhanced through adapted Ca²⁺ signaling, facilitated through increased endothelial connexin 43 (Cx43) gap junctional communication (GJC). In preeclampsia (PE), this adaptive response is missing. Of note, the angiogenic factor VEGF can also act via Src and ERK to close Cx43 gap junctions. While VEGFR2 is necessary for such closure, a role VEGFR1 is less clear. We reasoned if VEGFR2 is acting alone, then substituting another growth factor receptor with VEGFR2-like signaling should have the same effect. In uterine artery endothelial cells derived from pregnant sheep (P-UAEC), endogenous EGFR expression is very low. When we used adenovirus to raise EGFR, we also dose-dependently induced EGF-sensitive Cx43 phosphorylation mainly via ERK, and corresponding loss of Ca²⁺ bursts, but eliminated VEGF effects on phosphorylation of Cx43 or loss of Ca²⁺ bursting. This surprising observation suggests that while activated EGFR may indeed substitute for VEGFR2, it also sequesters a limited pool of effector molecules needed for VEGFR2 to phosphorylate Cx43. Thus, low endogenous EGFR expression in P-UAEC may be a necessary strategy to allow VEGFR-2 control of GJC, a first step in initiating angiogenesis in healthy pregnancy. Of further

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Declaration of Interest

All authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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note, trophoblasts are rich in EGFR, and we have demonstrated shed PLAP+/EGFR+ extracellular vesicles in maternal circulation in first trimester plasma samples using nanoscale high resolution flow cytometry. Collectively our data suggest that placenta derived exosomes positive for EGFR should be further considered as a possible cause of endothelial dysfunction in women with PE.

Keywords

EGF; EGFR; VEGF; Pregnancy; Preeclampsia; Gap junction; Endothelium; Exosome; Src; ERK; Ca²⁺

1. Introduction

During pregnancy, the cardiovascular system must undergo sweeping changes to accommodate the needs of mother while also supporting the developing fetus. Soon after implantation, the maternal vasculature begins to exhibit increases in heart rate, stroke volume, plasma and red blood cell volume (Reviewed in Sanghavi and Rutherford, 2014). However, the increased cardiac output and blood volume are not associated with hypertension, as they would otherwise be in the nonpregnant state, because there is a concomitant fall in vascular resistance. Early in pregnancy, this is predominantly due to increased uterine angiogenesis, while later the dominant mechanism is sustained vasodilation. We have coined the term ‘pregnancy-adapted programming’ to refer to events at the level of cell signaling that underlie enhanced function of the maternal vascular endothelium such that systemic vasodilation in general and uterine vasodilation in particular are sufficiently enhanced to reduce vascular resistance and divert blood to the uterus, so meeting the increased demands of the fetus.

Diseases of pregnancy such as intrauterine growth restriction and preeclampsia are in turn characterized by a failure of these adaptations (Reviewed in Li et al., 2018). Preeclampsia (PE) is also known to be a state of altered angiogenic factors, including placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) (Reviewed in Hod et al., 2015). The ability of VEGF to downregulate Ca²⁺ bursts necessary for vasodilator production through VEGF receptor-2 (VEGFR2) is dependent on Src- and ERK-mediated phosphorylations of Cx43 at Tyr-265 and Ser-279/282, respectively, and pharmacological inhibition of Src or MEK/ERK signaling protected Ca²⁺ bursting from the inhibitory effects of VEGF (Boeldt et al., 2015).

The VEGF-VEGFR system is a family of numerous variants of both growth factor and receptor. This is not helped by the fact that the tools to study the system (e.g. VEGFR kinase inhibitors or subtype selective binding ligands) are very limited. While VEGF-E activation of VEGFR2 alone appears capable of inhibiting GJC, PlGF activation of VEGFR1 alone does not (Boeldt et al., 2015). Still, a role for activated VEGFR1/R2 heterodimers cannot be ruled out. Interestingly, the epidermal growth factor (EGF) is also known to activate Src and MAPK pathways, and its single receptor—EGFR—is far better characterized than VEGFR1 and 2. Due to decades of well-funded cancer research, an extensive assortment of tools has been developed for its study. A wide variety of pharmacological EGFR inhibitors are commercially available, as are fluorophore-tagged EGFR ligands. Dozens of highly selective

antibodies that target specific sites on EGFR have been developed, including reliable antibodies for each of the many distinct phosphotyrosine sites. Site directed mutagenesis of particular EGFR phosphotyrosine sites can be strategically employed to eliminate the receptor's ability to activate specific signal transduction pathways. Furthermore, a plethora of naturally occurring and artificially created EGFR mutants have been characterized. There are dozens of identified missense mutations, deletion mutations, and duplication mutations—some that increase constitutive kinase activity and ligand sensitivity (e.g. L834R) (Shan et al., 2012), and some that eliminate ligand dependency altogether (e.g. EGFRvIII) (Pedersen et al., 2004). There are also mutants that exhibit impaired tyrosine kinase activity (K721M) (Chen et al., 1987; Honegger et al., 1987).

Given that EGFR can signal through Src- and ERK-mediated pathways activated by VEGFR2, then we proposed that simple overexpression of EGFR in pregnancy-adapted uterine artery endothelial cells (P-UAEC) should also enable the EGF-EGFR system to drive PE-associated endothelial dysfunction in a similar manner as does the VEGF-VEGFR system. More specifically, we predicted (1) that overexpression of EGFR in P-UAEC at a level sufficient to induce constitutive activity (or expression of a constitutively active EGFR mutant) could result in a permanent basal inhibition of vasodilatory mechanisms, thus simulating the functionally impaired state of the vascular endothelium in PE; (2) that even if constitutive downregulation of endothelial cell function were not achieved, overexpression of EGFR in P-UAEC would likely enable EGF treatment to inhibit the molecular mechanisms of vascular adaptation to pregnancy in P-UAEC just as VEGF treatment does; and (3) that placental exosomes may well be a source of EGFR that could fuse with endothelial cells and so precipitate similar effects in adverse pregnancy. Our findings presented here largely support this proposal, and indeed the possibility that EGFR transfer may occur, but with important caveats. Our studies also reveal unexpected evidence that signaling molecules in P-UAEC may be rate limiting and when EGFR is elevated, competition can occur to the deficit of the VEGFR family. The relevance of these findings to PE are discussed.

2. Materials and Methods

2.1. Materials

Unless noted otherwise, MEM and all other cell culture reagents were purchased from Life Technologies (Carlsbad, CA). Adenoviruses [Ad-EGFR(ErbB1) and Ad-EGFR(L834R)] were generated by Vector Biolabs (Malvern, PA) to encode the wild-type epidermal growth factor receptor [*Homo sapiens*] and its L834R mutation, respectively. Human recombinant epidermal growth factor (EGF) and CaCl₂ were from EMD Millipore (Temecula, CA). ATP (disodium salt), PP2 and all other chemicals, unless noted otherwise, were from Sigma (St. Louis, MO). Vascular endothelial growth factor (VEGF-A₁₆₅ - referred to from here on as VEGF) was from R&D Systems, Inc (Minneapolis, MN). U0126 was from Promega Corp (Madison, WI). For flow cytometry studies, Mab528 (EGFR-selective antibody) was from EMD Millipore. Mouse IgG2a (isotype control) and R-phycoerythrin goat anti-mouse IgG (H+L) were from Molecular Probes (ThermoFisher Scientific, Waltham, MA). For Ca²⁺

imaging studies, 35-mm dishes with glass coverslip windows were purchased from MatTek Corp (Ashland, MA). Fura-2 AM and Pluronic F127 were obtained from Life Technologies.

2.2. Isolation of uterine artery endothelial cells

Procedures for animal handling and protocols for experimental procedures were approved by the University of Wisconsin-Madison Research Animal Care Committees of both the School of Medicine and Public Health and the College of Agriculture and Life Sciences and followed the recommended American Veterinary Medicine Association guidelines for humane treatment and euthanasia of laboratory farm animals. Uterine arteries were obtained from mixed Western breed pregnant (P) ewes at 120–130 days of gestation during non-survival surgery, and UAECs were prepared by collagenase dispersion as previously described (Bird et al., 2000; Grummer et al., 2009). Cells were routinely grown and passaged in MEM based growth medium containing 20% FBS, 1% penicillin-streptomycin, and 4 ug/ml gentamycin. Cells collected at the end of passage 3 were then stored at a 1:4 split in liquid nitrogen.

Pools of UAEC were then created for study use by growing $1 \times T75$ of cells at passage 3 from 4 different pregnant ewes to near confluence, and combining all cells into a pool before freezing down at a 1:35 split ratio for Ca^{2+} imaging studies or 1:4 for Western blot and FACS studies. Prior to creating the pool, we had previously verified cells from each ewe exhibited a 'pregnancy adapted' (ie enhanced/sustained) Ca^{2+} response to 100uM ATP relative to cells from nonpregnant ewes, and the response of the cell pool to ATP stimulation was equivalent to the averaged response of source cells from the 4 individual animals.

For imaging studies, cells from one vial of pooled cells (1:35) were distributed to 6 glass-bottom dishes of cells in 20% growth medium as above. On Day 4 of incubation, the Adenoviral Transduction Protocol was then begun.

For flow cytometry and Western blot studies, a cells from single vial (1:4) was seeded and grown to near confluence in a T75 flask. After reaching 80–90% confluency, cells were trypsinized and seeded to the wells of 2×6 -well plates at a density of 350,000 cells per well, and maintained overnight before the Adenoviral Transduction Protocol was begun (see below).

2.3. Adenoviral Transduction Protocol

Note whether we used cells for Ca^{2+} imaging in glass bottom dishes or used cells for Western or FACS studies in 6 well plates, all dish/well sizes, adenovirus dilutions, and incubation volumes were equivalent.

2.3.1 For Western blot and Conventional Flow Cytometry experiments: *Day 1:* P-UAEC grown to 80–90% confluency in T-75 flasks were recovered and seeded to 6-well plates containing 2 mL Minimum Essential Medium (MEM) w/20% fetal bovine serum (FBS) medium and incubated overnight at 37°C. A new vial of cells was used for each additional independent experiment. *Day 2:* Medium was aspirated from dishes/wells, and 1 mL of control medium or adenovirus-containing medium was added to each well as appropriate. Adenoviral transduction medium was prepared by adding adenovirus stock [Ad-

EGFR(ErbB1) or Ad-EGFR(L834R) to medium at the desired multiplicity of infection (MOI), which was determined by titer. Cells were then incubated for 24 hrs at 37°C. *Day 3:* Medium was aspirated from wells, and 2 mL of fresh MEM (w/20% FBS) was added to each well, which were incubated overnight at 37°C. *Day 4:* Cells typically achieved near confluence (~90–100%) prior to experimental treatments (Flow cytometry or Western blot).

2.3.2 For Ca²⁺ imaging experiments: *On Day 4* (Plating of cells in glass-bottom dishes having occurred on Day 1): Medium was aspirated from glass-bottom dishes, and 1 mL of control medium or adenovirus-containing medium was added to each well as appropriate. Adenoviral transduction medium was prepared by adding adenovirus stock [Ad-EGFR(ErbB1) or Ad-EGFR(L834R)] to medium at the desired multiplicity of infection (MOI), which was determined by titer of antivirus and estimation of cell density. Cells were then incubated for 24 hrs at 37°C. *Day 5:* Medium was aspirated from wells, and 2 mL of fresh MEM (w/20% FBS) was added to each well, which were incubated overnight at 37°C. *Day 6:* Cells typically achieved confluence of ~90–100% were used for Ca²⁺ imaging.

2.4. Conventional Flow Cytometry Protocol for P-UAEC

The Adenoviral Transduction Protocol was used with adenovirus that contains the wild-type *EGFR* or *L834R-EGFR* gene at multiplicities of infection (MOIs) of 0, 10, 100, 320, 1000, and 3200 in 6-well plates. A431 cells, which are known to express high concentrations of EGFR protein on the plasma membrane, were also cultured to serve as a robust positive control. After the Adenoviral Transduction Protocol was completed as described, medium was aspirated from all wells and cells were lifted with 1 ml of trypsin. Cells were spun down at 300g for 3 min, supernatant was aspirated, and each sample of cells was resuspended in 1 ml MEM with 2% (*not* 20%) FBS. 100 uL of suspended cells from each of the 6 tubes were then pipetted into TWO 1.5 ml Eppendorf tubes on ice at 40C. For each set of samples, 14 tubes each containing 100 uL of cells were labeled 1a, 1b, 2a, 2b, ..., 7a, and 7b. To all the “a” tubes, 10 uL of murine IgG2a diluted 1:10 in PBS was added. To all the “b” tubes, 10 uL of mAb528 diluted 1:10 in PBS was added. Samples were mixed gently and incubated on ice for 30 min. Following incubation, 1 ml MEM w/2% FBS was added to each tube and spun in a microcentrifuge at 300g for 3 min. Supernatant was aspirated from each tube, 90 uL fresh MEM w/2% FBS was added to each tube, and 10 uL of R-phycoerythrin fluorescent tagged goat anti-mouse IgG (H+L) diluted 1:10 in PBS was added to all 14 tubes. Tubes were then incubated on ice for 30 min as before. Following this second incubation, 1 ml MEM w/2% FBS was added to each tube and spun in a microcentrifuge at 300g for 3 min. Supernatant was again aspirated from each tube, 300 uL PBS was added to each tube, and cells were gently resuspended. Each sample was transferred to a separate 5 mL clear plastic flow cytometry tube and kept on ice. Samples were analyzed by the BD FACS Calibur flow cytometer at the Flow Cytometry Laboratory at the Wisconsin Institutes for Medical Research (WIMR). One drop of propidium iodide solution was added to each tube before analysis to identify dead cells. For each sample, the fluorescence intensity of 10,000 cells was quantified (which served as an indicator of plasma membrane expression of EGFR) and the geometric mean was calculated. Mean fluorescence intensity of matching counterparts from the two sets were averaged to produce 14 final mean values—7 for EGFR-

selective binding of mAb528 and 7 for the corresponding IgG controls for A431 cells and P-
UAEC at MOIs of 0, 10, 100, 320, 1000 and 3200.

2.5. Western Blot Protocol

Pooled P-UAEC grown in T75 flasks were passaged to 6-well plates (350,000 cells per well) and maintained overnight in MEM w/20% FBS to adhere securely to the well at approximately 50–55% confluency. At 48 hrs before agonist or growth factor treatment, the Adenoviral Transduction Protocol was performed on all wells, using either vehicle or adenovirus containing the desired gene (*wtEGFR* or *L834R-EGFR*). At 4 hrs prior to treatment with either agonist or vehicle, cells were incubated in MEM with 0.01% BSA without FBS. Pharmacological inhibitors were added 20 min prior to 30-min growth factor treatment, as appropriate. Reactions were terminated by immediate removal of media and snap-freezing in liquid N₂. Cells were solubilized in lysis buffer [4 mM Na(PO₄)₂, 50 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM EDTA, 10 mM NaF, 2 mM Na₃(VO₄)₂, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 ug/ml leupeptin, and 5 ug/ml aprotinin], sonicated, and centrifuged at 12,000 × g for 5 min. Solubilized protein concentration was determined using the bicinchoninic acid assay (Sigma, St. Louis, MO), which was followed by Western blotting (20 ug protein per lane) on 7.5% polyacrylamide gels (100 V, ~2 h; Mini- Protein II, Bio-Rad Laboratories Inc., Hercules, CA) and transfer to Immobilon P membrane (100 V, 2 hrs; Millipore, Billerica, MA). Starting Block TBS blocking buffer (ThermoFisher Scientific, Waltham, MA) was used for blocking all membranes. For each experiment, four blots were run together in two pairs/gel rigs to ensure consistent data across membranes.

For each trial, proteins were detected by first probe of 4 replicate membranes using the following antibodies: pS279/282 Cx43 (#sc-12900-R; Santa Cruz Biotechnology, Santa Cruz, CA) 1:667; pY265 Cx43 (#sc-17220-R; Santa Cruz) 1:667; total Cx43 (#sc-9059; Santa Cruz) 1:2000; ERK1/2 pT202/pY204 (#4377S, Cell Signaling) 1:1000; total ERK1/2 (#9102S, Cell Signaling) 1:2000; Akt pS473 (#9271L, Cell Signaling) 1:1000; total Akt (#9272, Cell Signaling) 1:2000; pY1173 EGFR (#44–794G, Invitrogen) 1:1000; total EGFR (#sc-03, Santa Cruz) 1:1000. Protein bands were normalized on each membranes second use to levels of Hsp90 (#4874S, Cell Signaling) 1:2000. For phosphorylation-state specific antibodies we used goat anti-rabbit HRP-conjugated secondary antibody (# 7074; Cell Signaling Technology, Inc., Danvers, MA) at 1:4000. For Akt pS473 and Cx43 pS279/282 and pY265, specific binding was detected by enhanced chemiluminescence (ECL) 2 reagent detection system (GE Healthcare BioSciences, Piscataway, NJ); ECL was used for all other proteins. Bands were quantified using the Epson 4990 Photo scanner in transmission scanning mode (Epson America, Long Beach, CA) and band density determined using ImageJ software (version 1.50i, Wayne Rasband, National Institutes of Health, USA).

Normalization of data was achieved using second probing of all membranes for HSP90 for two reasons. First, the expression levels of ERK and Akt in these cells are unusually high and antibodies for total Akt and ERK1/2 tended to produce such strong bands that we had to either use suboptimal concentrations of antibody or exposures were so short that data between experiments were inconsistent (due to the risk of over exposure). The second reason

is that several of these phosphoproteins fall into molecular weights at or below 40Kd, and in our experience re-probing membranes involves some loss of low molecular weight proteins (about 10%). So the data from first probing is not actually normalized unless we use a higher molecular weight protein in round two that is more strongly associated with the membrane.

2.6. Fura-2 Ca²⁺ imaging studies

Pooled passage 4 P-UAEC on 35 mm glass-bottom microwell dishes were prepared for imaging by washing and then incubating in 1 ml Krebs buffer (125 mM NaCl, 5mM KCl, 1mM MgSO₄, 1mM KH₂PO₄, 6mM glucose, 25mM HEPES, 2mM CaCl₂, pH 7.4) at 37°C. Cells were loaded with Fura-2AM (10uM) in the presence of 0.05% Pluronic F127 for 60 min. P-UAEC were washed again with Krebs buffer and incubated for 30 min to allow completion of ester hydrolysis (as in Yi et al. (2010b)). Fura-2 loading was verified by viewing at 380 nm UV excitation on a Nikon inverted microscope (InCyt Im2, Intracellular Imaging, Inc., Cincinnati, OH, USA). The cells were (1) treated with ATP (100uM) for 30 min, (2) rinsed and given fresh buffer, treated with PP2 or U0126 (10 uM), or vehicle for 30 min, (3) treated with VEGF or EGF (10ng/ml), or vehicle for 30 min, and (4) stimulated a second time with ATP (100 uM) for 30 min. During steps 1,3 and 4, the data was recorded for 80–100 cells simultaneously using alternate excitation at 340 and 380 nm at 1-s intervals and measuring emitted light using a PixelFly video camera (Cooke). Data were recorded for a minimum of 5 minutes before ATP stimulation or growth factor addition to establish the basal free [Ca²⁺]. Ca²⁺ bursts were counted for the 1st and 2nd ATP treatments. Those that gave 3 or more bursts during the initial ATP treatment were included in the data set and the others were discarded. Mean number of bursts for each cell following the 2nd ATP treatment were expressed as a percentage of the mean number of bursts following the 1st ATP treatment. The number of replicates (“n”) for each growth factor or kinase inhibitor treatment group equals the total number of responding cells in each group aggregated from 5 – 22 independent trials.

2.7. Human plasma samples for extracellular vesicle analysis

Platelet poor plasma was prepared from pregnant women using OHSU IRB approved protocols (#10744; #15560). Briefly, antecubital vein blood samples were collected in EDTA tubes at room temperature from 36 pregnant women at 6–10 weeks’ gestation (mean 7.8 weeks +/- 0.26) and four adult male negative controls. Maternal age averaged 28.5 years +/- 1.1 years, most were nulligravida, race was non-Hispanic white, they were non-obese, and they were not smokers; fetal sex was unknown for this study. Gestational age was determined by crown-rump length on trans-vaginal ultrasound to confirm intra-uterine pregnancy. Fresh blood samples were centrifuged at 2500 x g for 15 minutes (brake on low); the supernatant was then spun again at 2500 x g for 15 minutes to generate platelet poor plasma, which was dispensed into 100uL aliquots, flash frozen, and banked at -80 C. Importantly, all of the blood samples tested in this study were uniformly collected, centrifuged within 2 hours, and banked. Platelet depletion was confirmed by conventional flow cytometry labeling with CD41.

2.8. Nanoscale high resolution flow cytometry

Antibodies used for extracellular vesicle (EV) flow cytometry required high purity and uniform binding kinetics established by comparing the results of room temperature incubations for one hour to overnight incubations at 4°C. Anti-PLAP-APC (eBiosciences), EGFR-PE (described above), and CD63-FITC (BD Biosciences) optimal dilutions (0.2 µg/ml to 0.02 µg/ml) were determined by testing dilution series in positive and negative control plasma samples (e.g. pregnant plasma versus male for PLAP) and by staining EV depleted plasma that was treated overnight with 5% DMSO at room temperature (negative control). Plasma samples were stained with multiplex cocktails of all three antibodies for 1h at room temperature then diluted 1:100 into 200nm bead buffer ([Polysciences.com](https://www.polysciences.com)) created by FACS sorting 200nm beads into 0.1 µm filtered PBS. 200nm bead buffer provided both an internal size standard and a means to standardize the volume of stained plasma tested (e.g. 1000 beads equaled 1 µl of starting plasma tested on our FACS Aria Fusion (BD Biosciences)). Nanoscale flow cytometry imaging of stained EVs was performed as described by our group and others (Kormelink et al., 2016; Morgan 2018; Mitrugno et al., 2019). Briefly, the approach employed the combination of both side light scatter (SSC-H) reported on the log scale of the y-axis and antibody-fluorophore labeling reported on the x-axis, which distinguished EV relative sizes (compared with commercially available polystyrene bead standards ranging in size from 100nm-900nm, Megamix-Plus FSC and -Plus SSC, BioCytex) and multiplex antibody target specificity. Labeled EVs were distinguished from the unlabeled nanoparticle “noise” of similar size (e.g. lipoproteins, non-specific cell fragments) by positive antibody staining fluorescent signal. Fluidic and instrument settings were designed to minimize background noise while maximizing the detection of submicron sized EVs. SSC-H voltage was uniformly adjusted for all experiments so 100nm Megamix beads were at SSC 10^3 , 200nm beads were at SSC 10^4 , 500nm beads were at 10^5 , and 900nm beads remained on scale. All flow machine buffers were filtered with a ZenPure PureFlow Mini Capsule PES 0.1 µm filter. Imaging was performed on a FACS Aria Fusion (BD Biosciences) with a 70 µm nozzle and 0.1 µm filtered PBS sheath fluid at a pressure of 70 psi. Coincident EV events per nanoliter droplet were controlled by using a threshold rate of <40,000 events per second and electronic abort rate less than 5%. An essential criterion for reproducibility was setting uniform gates in advance of batch analysis with positive and negative control plasma samples. All samples used the same pre-set gates defined relative to MegaMix beads and plasma controls. Data were collected based on uniform 200nm bead counts spiked into each sample to standardized plasma volumes being tested (data reported as counts/µl of plasma). The mean of triplicate experiments for each sample was employed for statistical analysis comparing PLAP, EGFR, CD63 and co-labeled EV events (e.g. PLAP/EGFR) between first trimester plasma and male plasma negative controls.

2.9. Statistical analysis

For each Fura-2 Ca²⁺ imaging experiment, fluorescent signal was averaged from at least 20 responding cells. Imaging data were from 5 to 22 separate dishes from each treatment group. For other assays, 4–9 independent experiments were performed. Data are presented as box-and-whisker plots or columns indicating means ± S.E.M. For Figs. 1C, 3B, 4A, 4B, 4C, 5B, 6C, and 7A, statistical significance was determined by ANOVA and Tukey’s HSD post-hoc test. For Fig. 5A, Welch’s t-test for comparison of two samples with unequal variances

was used because variance of Akt phosphorylation was considerably higher in adenovirally transduced cells. For Fig. 6A, significance was determined by unpaired t-test. For Figs. 1B, 6B, and 7B, the Wilcoxon Rank-Sum test was used for each sample against vehicle control. For Fig 8, EV counts were compared within and between groups (first trimester vs male controls) by ANOVA. A value of $P < 0.05$ was considered statistically significant (* < 0.05 , ** < 0.01 , *** < 0.001).

3. Results

3.1. EGFR expression and Cx43-related activity in P-UAEC

3.1.1. EGFR is weakly expressed in P-UAEC—Flow cytometric quantification of EGFR expression on the plasma membrane was achieved by the use of mAb528, which has a similar affinity for the extracellular ligand binding domain as does EGF without promoting rapid internalization (Reilly et al., 2000). Using this method, we detected the receptor at very low levels on the surface of P-UAEC in 22% of cells (Fig. 1A), consistent with previous immunocytochemical staining of P-UAEC (Bird et al., 2000).

3.1.2. Effects of EGF and VEGF on Ca²⁺ bursting and Cx43 phosphorylations in P-UAEC—The mean number of ATP-stimulated Ca²⁺ bursts were compared before and after P-UAEC were treated with EGF or VEGF (10 ng/mL). While VEGF reduced the mean number of ATP-induced Ca²⁺ bursts compared to the cells treated with vehicle ($P < 0.0001$), EGF treatment results were not significantly different than the control group (Fig. 1B). As showed in prior published work (Boeldt et al., 2015), 30-minute VEGF treatment of P-UAEC reduced Ca²⁺ burst numbers to the level observed in UAEC derived from nonpregnant ewes, an effect dependent on VEGFR-2 signaling through Src and ERK.

3.1.3. Effects of EGF and VEGF on phosphorylations of Cx43 Y265 and S279/282—While VEGF induces phosphorylations of Cx43 at Y265 and S279/282 (Boeldt et al. (2015)), here we found that in P-UAEC not yet exposed to adenovirus Ad-EGFR, EGF does not appear to significantly elevate the phosphorylation of either site (Fig. 1C), an observation consistent with EGF's inability to downregulate Ca²⁺ bursting. In contrast, the VEGF positive control induced a 1.5-fold elevation of pY265 ($P = 0.029$), similar to the value previously reported in Boeldt et al. Unfortunately, due to degradation of performance of the commercial anti-pS279/282 antibody, we were not able to reproduce the previously reported observation of elevated pS279/282 following VEGF treatment in P-UAEC, nor were we able to replace the antibody from another source.

3.2. Expression of exogenous EGFR in P-UAEC via adenoviral transduction

EGFR protein was undetectable by Western blot in P-UAEC not exposed to adenovirus (now termed 'parental P-UAEC') and in cells transduced at an MOI of up to 10. However, EGFR was clearly observed by FACS and Western blot at an MOI of 100, and was found to be robust at an MOI of 1000 (Fig. 2A). We also transduced P-UAEC with adenovirus containing the *L834REGFR* gene, which encodes for one of the most common constitutively active EGFR mutants found in non-small cell lung cancer. Experiments with these cells—

termed 'P-UAEC-adL834R'—became trivial when it became apparent that they exhibited responses to EGF that were virtually identical to cells transduced with wild-type *EGFR*. The experimental data obtained from these cells is presented as a supplement to this publication.

Because Western blotting does not distinguish between cytosolic and surface expression of proteins, we utilized parallel flow cytometry analysis to obtain a more precise measurement of EGFR expression specifically at the plasma membrane. When samples of parental P-UAEC were subjected to the immunolabeling protocol and subsequent analysis, including pretreatment with propidium iodide to stain dead cells, approximately 80% of the 10,000 cells that passed through the FACS machine survived. At the completion of the protocol, cell viability remained at approximately 80%, suggesting that neither exposure to adenovirus nor expression of exogenous EGFR had any significant deleterious effect on cell viability (data not shown). The flow cytometry data confirmed that the percentage of P-UAEC that expressed EGFR on the cell surface increased as a function of MOI. At an MOI of 3200, 100% of P-UAEC expressed detectable EGFR, though the level of expression varied widely from cell to cell (Fig. 2B). By comparing the mean of fluorescence intensity of each MOI treatment group of P-UAEC to that of A431 cells (data not shown), which are known to express $\sim 2.6 \times 10^6$ EGFR per cell (Haigler et al., 1978), we also obtained the first ballpark estimation of the number of EGFR molecules on the plasma membrane of parental (MOI 0) and adenovirally transduced (MOI 10–3200) P-UAEC (Table 1). The low receptor numbers in parental P-UAEC (~ 7000 EGFR/cell) are consistent with its near undetectability by Western blot and the lack of observed effects of EGF on Cx43 GJC. Adenoviral transduction at MOIs of 1000 and 3200 increased expression 26-fold and 75-fold, respectively. Of note, increases in EGFR expression remained linearly proportional to MOI from 0 to 3200, suggesting no loss of mAb528 binding at higher MOIs. Some degree of additional verification was provided by Western blot where expression appeared to increase 110-fold at an MOI of 1000, but with the caveat that EGFR expression at lower MOIs (i.e. 0, 10) was near the lower limit of film sensitivity. Accurate quantification of EGFR protein bands obtained from MOI 3200 cells was not possible due to film saturation.

3.3. EGFR in P-UAEC becomes constitutively autophosphorylated when transduced at an MOI of 1000

In the field of cancer research, it is well-established that EGFR becomes constitutively activated when overexpressed in cells at a sufficient level, thus aggressively driving the cell into a dysregulated state. In order to determine the minimum level of EGFR overexpression that resulted in constitutive receptor autophosphorylation, we performed western blot analysis to observe autophosphorylation of Y1173, one of the most studied tyrosine phosphorylation sites of the receptor. Treatment of cells with EGF, while not necessary for observing basal autophosphorylation, was done to provide a comparison of the magnitude of ligand-dependent and independent activity of EGFR. While P-UAEC in the MOI 100 group did exhibit visible autophosphorylation in response to 30-minute EGF treatment (Fig. 3A), only cells in the MOI 1000 group exhibited constitutive autophosphorylation ($P=0.001$) (Fig. 3B). No significant basal or ligand-responsive autophosphorylation of EGFR was observed in parental P-UAEC or the MOI 10 cells.

Having established that an MOI of 1000 achieved the EGFR expression level at which constitutive autophosphorylation occurred, this MOI was used for almost all subsequent experiments. Therefore, the phrase “P-UAEC that have been adenovirally transduced with the *EGFR* gene at an MOI of 1000” is termed “P-UAEC-adEGFR” from this point forward.

3.4. ERK1/2 phosphorylation in P-UAEC-adEGFR

While it is important to establish the ability of exogenous EGFR to signal through MEK/ERK and Src pathways, limited antibody sensitivity in ovine cells prevented us from directly measuring the effect of EGFR overexpression on Src phosphorylation in P-UAEC-adEGFR. We were, however, able to quantify the basal and EGF-induced phosphorylation of ERK1/2 in P-UAEC-adEGFR.

3.4.1. Basal phosphorylation of ERK1/2 in P-UAEC is not significantly increased in P-UAEC-adEGFR—To quantify basal phosphorylation of ERK1/2, we performed western blot analysis in both parental P-UAEC and P-UAEC transduced with the *EGFR* gene at adenoviral MOIs of 10, 100, and 1000. ANOVA revealed no significant differences in ERK phosphorylation between any cells for a given MOI. However, a distinct *trend* of greater pERK1/2 with increased MOI was observed, suggesting the possibility of basal elevation of ERK-mediated Cx43 S279/282 phosphorylation at higher MOIs. Cells transduced at an MOI of 1000 (P-UAEC-adEGFR) exhibited the greatest increases of mean basal ERK1 and ERK2 phosphorylations at 2.1- and 1.9-fold, respectively, above the levels observed in parental P-UAEC, but these elevations were not found to be significant due to the high variance between trials (Fig. 4A).

3.4.2. Basal phosphorylations of Cx43 Y265 and S279/282 in P-UAEC are not significantly increased in P-UAEC-adEGFR—If our hypothesis that overexpression of EGFR in P-UAEC would induce constitutive kinase mediated inhibition of GJC is correct, we would expect to see basal elevations of Cx43 pY265 and pS279/282. However, no change in the basal levels of phosphorylation of these residues was observed at MOIs of 10, 100, or 1000 (Fig 4B). Although we could not measure the phosphorylation of c-Src directly, basal c-Src phosphorylation is not likely to be significantly increased by the constitutive activity of EGFR in P-UAEC-adEGFR. This is because cells transfected with v-Src, the constitutively active viral form of Src, exhibit downregulated GJC and display basal elevation of Cx43 Y247 and Y265 (Swenson *et al.* 1990; Solan and Lampe 2008), and activated c-Src has the same inhibitory effects on GJC as v-Src (Azarnia *et al.* 1988).

3.4.3. Comparative effects of EGF and VEGF on ERK1/2 phosphorylation in P-UAEC-adEGFR—Despite the absence of significant elevation of basal ERK1/2 phosphorylation in P-UAEC-adEGFR, treatment with EGF induced robust phosphorylations of ERK1 ($P = 0.001$) and ERK2 ($P = 0.001$) at several times the levels observed in control cells, thus confirming a functional coupling to the MAPK pathway. However, in contrast with prior data showing significant phosphorylation of ERK1/2 by VEGF in parental P-UAEC (Bird *et al.*, 2000; Grummer *et al.*, 2009), administration of VEGF in P-UAEC-adEGFR at the same dosage and treatment duration had no effect on ERK1 or ERK2 phosphorylation (Fig. 4C).

3.5. Comparative effects of EGF and VEGF on Akt phosphorylation in parental P-UAEC and PUAEC-adEGFR

Because Akt is known to regulate the size and stability of gap junctional plaques (Dunn et al., 2012; Dunn and Lampe, 2014), we performed western blot analysis to quantify the effects of EGF and VEGF on the phosphorylation of Akt S473, one of two known activation sites, in parental P-UAEC and P-UAEC-adEGFR.

3.5.1. Overexpression of EGFR in P-UAEC increases Basal phosphorylation Akt S473—In contrast to basal ERK phosphorylation, which resists persistent elevation from constitutive EGFR activity in P-UAEC-adEGFR, the mean level of basal Akt pS473 was elevated more than 16-fold ($P = 0.003$) above the basal level in parental P-UAEC (Fig. 5A).

3.5.2 EGF treatment induces Akt S473 phosphorylation in P-UAEC-adEGFR, but VEGF does not—While there appeared to be a trend toward greater Akt S473 phosphorylation following EGF treatment compared to vehicle control, the effect was not found to be significant due to high variance (Fig. 2B, left). In P-UAEC-adEGFR, EGF raised S473 phosphorylation 2.6-fold ($P = 0.003$) over the already elevated basal level (equivalent to 44-fold of basal level in parental PUAEC), suggesting that the capacity for Akt activation far exceeds typically observed physiological levels (Fig. 2B, right). In contrast, VEGF treatment had no observable effect on Akt phosphorylation in P-UAEC or P-UAEC-adEGFR. This is consistent with our prior data suggesting that neither VEGFR-1 nor VEGFR-2 is functionally linked to the PI3K/Akt pathway in P-UAEC (Grummer et al., 2009), unlike the known ability of VEGFR2 to activate this pathway in other endothelial cells and many other cell types (Reviewed in Karar and Maity, 2011).

3.6. Effects of constitutive and EGF-induced EGFR activation on the Ca²⁺ burst response to ATP in P-UAEC-adEGFR

3.6.1. Basal EGFR activity does not constitutively downregulate Ca²⁺ bursting—If our hypothesis were correct that overexpression of EGFR in P-UAEC would have a permanent constitutive inhibitory effect on GJC, we would expect treatment with ATP to result in a significantly weaker Ca²⁺ bursting response in P-UAEC-adEGFR than in parental P-UAEC. By aggregating the data from every calcium imaging trial, we were able to compare the mean number of ATP-stimulated [Ca²⁺]_i bursts exhibited by parental P-UAEC vs. P-UAEC-adEGFR prior to any administration of kinase inhibiting drugs or growth factors. Contrary to our prediction, we observed no reduction in the mean number of Ca²⁺ bursts. Surprisingly, the mean number of bursts per cell was actually 4% greater ($P = 0.005$) in P-UAEC-adEGFR than the mean number of bursts in parental P-UAEC (Fig. 6A). This observation was inconsistent with our hypothesis that the constitutive kinase activity of overexpressed EGFR would induce a permanent basal inhibition of GJC.

3.6.2. EGF treatment of P-UAEC-adEGFR downregulates Ca²⁺ bursting to a level consistent with VEGF in parental P-UAEC.—In P-UAEC-adEGFR, the results were reversed from those seen in parental P-UAEC. Treatment with EGF now induced significant inhibition of ATP-induced Ca²⁺ bursting. Unexpectedly, VEGF treatment no

longer had an effect (Fig. 6B), precisely the opposite of the respective effects of EGF and VEGF observed in cells that normally don't express exogenous EGFR. Although the overexpression of EGFR in P-UAEC was shown to be insufficient to result in constitutive inhibition of GJC, the presence of exogenous EGFR clearly makes dramatic differences in the effects of transient growth factor treatment on gap junction function.

3.6.3. Comparative effects of EGF and VEGF on phosphorylations of Cx43 S279/282 and Y265 in P-UAEC-adEGFR—

In Figure 1C, Western blot analysis showed that phosphorylation of the Cx43 inhibitory site Y265 was significantly increased 1.5-fold by 30-minute treatment with VEGF, but not by EGF treatment. This is consistent with the results of calcium imaging experiments using parental P-UAEC, in which VEGF treatment significantly reduced mean number of ATP-induced Ca²⁺ bursts, but the EGF treatment group results were no different than the control group (Fig. 1B). However, in P-UAEC-adEGFR, EGF treatment resulted in a 1.4-fold increase in Y265 ($P = 0.044$) and a 2.9-fold increase in S279/282 ($P = 0.001$) (Fig. 7B), while 30-minute treatment with VEGF produced no significant effect at either Cx43 phosphorylation site. It should be noted that the anti-Cx43 pS279/282 antibody was able to detect the *supraphysiological* level of EGF-stimulated pS279/282 in P-UAEC-adEGFR despite the antibody's overall loss of selectivity.

3.7. Effects of pharmacological inhibition of Src or MEK on EGF-mediated downregulation of Ca²⁺ bursting in P-UAEC-adEGFR—

Following the initial 30-minute stimulation with ATP but before EGF treatment, P-UAEC-adEGFR were pretreated with the Src family kinase-selective inhibitor PP2 or the MEK-selective inhibitor U0126. In Figure 7A, the effects of both pretreatments on Cx43 Y265 phosphorylation were shown to be just as expected. U0126 pretreatment prior to EGF administration was not significantly different from EGF alone, and PP2 pretreatment kept Y265 phosphorylation at a level not significantly different from basal pY265. Furthermore, in Figure 8A, the effects of both pretreatments on Cx43 S279/282 phosphorylation were also shown to be just as expected. U0126 pretreatment prior to EGF administration indeed kept S279/282 phosphorylation at the basal level, and PP2 pretreatment was not significantly different from EGF alone.

Having confirmed that both pharmacological kinase inhibitors effectively prevented their respective EGF-induced phosphorylations of Cx43, we then needed to determine how their administration would affect the ATP-stimulated Ca²⁺ bursting response. As expected, when the cells were pretreated with U0126 prior to EGF treatment, the mean number of ATP-stimulated Ca²⁺ bursts remained at the level of untreated control cells; however, when the cells were pretreated with PP2 prior to EGF treatment, the mean number of ATP-stimulated Ca²⁺ bursts dropped to the level observed when EGF alone was added (Fig. 7B). As reported in prior published work (Boeldt et al., 2015) both U0126 and PP2 were able to protect the Ca²⁺ burst response from inhibition by VEGF, suggesting a single pathway involving both ERK1/2 and Src. However, here we see that PP2 provided no protection from inhibition by EGF ($P = 0.0005$).

3.8. Placental PLAP+/EGFR+ extracellular vesicles are released into the maternal circulation in the first trimester of pregnancy

Our strict adherence to uniform blood collection, processing, banking, validated staining protocols, standardized flow cytometer settings, uniform sample dilution buffer, collection of uniformly gated events from uniformly collected plasma volumes (based on 200nm bead internal standard counts), and batch analysis performed in triplicate provided low coefficients of variation and highly reproducible results between experiments using a FacsAria Fusion flow cytometer ($r=0.97$). Compared with immunostained 200nm bead buffer alone or male plasma negative controls, we observed significantly increased PLAP/CD63 positive and EGFR/CD63 positive EVs in first trimester plasma (Figure 8). Sample volumes assayed were standardized by internal 200nm bead counts spiked into the dilution buffer for each sample (Figure 8e, 200nm bead counts/ul of sample tested). Variance in first trimester PLAP/EGFR positive EVs was greater than PLAP/CD63 positive EVs despite lower mean EV counts/ul (500/ul versus 2000/ul, respectively). The cause of this underlying variance did not seem to be attributable to gestational age (6–10 weeks) although results seemed to vary more toward the end of the first trimester (data not shown). Sample size was insufficient to further explore gestational age-related variance and pregnancy outcome and fetal sex data were not available for these elective termination derived plasma samples, but a relationship between PLAP+ and EGFR+ staining was clear.

4. Discussion

We have shown in prior published work that treatment of P-UAEC with either EGF or VEGF stimulates mitogenesis and each induces phosphorylation of ERK (Bird et al., 2000). We have also established more recently that VEGF acts predominantly or exclusively through VEGFR2 to downregulate GJC in P-UAEC via Src- and ERK-mediated phosphorylations of Cx43 (Boeldt et al., 2015). Until now the effect of EGF on GJC in P-UAEC has not been formally investigated but may be relevant given the general role of EGF in promoting wound healing, cellular migration, and growth in a wide variety of other cell types. EGF has indeed been shown to inhibit GJC in many other cell models (Madhukar et al., 1989; Lau et al., 1992; Oh et al. 1993), and this effect was found to be dependent on phosphorylation of ERK (Kanemitsu and Lau, 1993) and associated with a serine phosphorylation of Cx43 (Lau et al., 1992). In contrast, a review of the literature on EGF and Cx43 over the past 30 years turned up only a single case of EGF-induced enhancement of GJC. However, the unique mechanism of this enhancement involved *translational* control of new Cx43 expression, not direct Cx43 phosphorylation (Rivedal et al., 1996).

In this study, our Ca²⁺ imaging data showed that EGF is not able to inhibit GJC in parental P-UAEC as it does in other cells. Given that the only known molecular target of EGF ligand is EGFR, which we have previously identified in P-UAEC via immunocytochemistry (Bird et al., 2000), this suggests endogenous receptor number is either very low or coupling to the signaling pathways that mediate inhibitory Cx43 phosphorylations is absent.

Here, for the first time, we confirmed our suspicion of low receptor expression using flow cytometry. Parental P-UAEC were shown to express only ~7000 EGFR/cell, which may explain why the receptor is relatively undetectable by Western blot without extreme

overexposure of the photo image. Given this low endogenous expression of EGFR and weak coupling, we predicted that overexpressing exogenous EGFR in P-UAEC to a level that conferred ligand-independent autophosphorylation (~180,000 EGFR/cell at MOI 1000, see Table 1) would increase basal inhibitory phosphorylations of Cx43 in our endothelial cell model ('P-UAEC-adEGFR'), thereby achieving a permanent downregulation of pregnancy-adapted Ca²⁺ burst signaling. If the expression of constitutively autophosphorylated EGFR failed to impair Cx43 gap junction function in a ligand-independent manner, we hypothesized that the higher concentration of EGFR on the plasma membrane might still enable EGF to duplicate in P-UAEC-adEGFR the inhibitory effect of VEGF on GJC in parental P-UAEC.

As anticipated, overexpression of EGFR in P-UAEC induced constitutive autophosphorylation. However, it did not result in the *permanent* loss of Cx43 gap junction function that we had predicted. Basal EGFR activity in P-UAEC-adEGFR was insufficient to alter Cx43 activity, as evidenced by no significant change in Cx43 Y265 or S279/282 phosphorylation, or in the mean number of ATP-stimulated Ca²⁺ bursts. Of note, we also observed no changes in growth/passage rate or morphology in P-UAEC-adEGFR, suggesting that P-UAEC are resistant to dysregulation of MEK/ERK signaling by *continuous* EGFR activity. However, added exogenous EGF treatment of P-UAEC-adEGFR did induce strongly significant increases in ERK1 and ERK2 phosphorylations at several times the levels observed in control cells, and caused robust secondary phosphorylations of Cx43 with reduced Ca²⁺ bursting, thereby indicating that acute ligand stimulation can override the cells' apparent compensatory downregulation of the MEK/ERK pathway.

Curiously, although P-UAEC-adEGFR did not exhibit significant elevation of basal ERK phosphorylation compared to parental P-UAEC, the cells displayed an almost 17-fold increase in the basal phosphorylation of Akt S473, indicating that unlike the MAPK pathway, the PI3K/Akt pathway is directly sensitive to constitutive EGFR signaling. In the temporal sequence of kinase regulation of Cx43 gap junction turnover described by Solan and Lampe (2016) as the "kinase program," Akt activation is associated with the aggregation of connexons into larger gap junctional plaques, which has the net effect of improving GJC. The effect of exogenous EGFR on Akt signaling in P-UAEC-adEGFR suggests a putative mechanism for the small but highly significant 4% increase in the mean number of ATP-stimulated Ca²⁺ bursts observed in 30 minutes in P-UAEC-adEGFR compared to parental P-UAEC. Nonetheless, this increase in Akt activation provided no protection against subsequent EGF-mediated phosphorylation of Cx43 or inhibition of GJC as measured by suppression of Ca²⁺ bursting. The complete role of PI3K/Akt signaling in the regulation of GJC is a subject for future studies.

We speculate that low endogenous EGFR expression in primary P-UAEC localizes the receptor to regions of the plasma membrane distinct from those occupied by VEGFR2, and so activated EGFR may activate a local subpool of ERK that is unable to access Cx43 in P-UAEC. Studies in other cell models have shown that, upon activation by GPCRs or RTKs, uniquely localized pools of ERK1/2 in the cytosol (Reviewed in Luttrell 2003) are indeed mobilized to specific cellular regions, including the nucleus, mitochondria, golgi, endosomes, and the plasma membrane (Reviewed in Wainstein and Seger 2016; Zennadi et

al., 2012). While our data only offer indirect support of this hypothesis in P-UAEC, it provides a plausible tentative explanation for why VEGF-induced ERK phosphorylation in P-UAEC is correlated with Cx43 phosphorylation and loss of GJC function (Boeldt et al., 2015), but EGF-induced ERK phosphorylation is not. It is also noteworthy that in P-UAEC both VEGF and EGF are equally capable of driving mitogenesis, which requires translocation of ERK to the nucleus, and not retention near the plasma membrane. If the low expression of EGFR is indeed a way to deny the receptor access to the specific pools of ERK1/2 that mediate Cx43 phosphorylation, then increasing receptor expression should facilitate access to those pools by spilling EGFR into formerly inaccessible regions of the plasma membrane and associated signaling molecules.

Regarding whether the elevated level of EGFR can fully recapitulate in P-UAEC-adEGFR the inhibitory action of VEGF in parental P-UAEC, the simple answer is in general EGFR can promote similar degrees of Cx43 phosphorylation and functional inhibition of bursting when EGF is also present, but with two surprising differences. Firstly, downregulation of Ca²⁺ bursting by EGF was reversed by the MEK inhibitor U0126, but not by the Src family kinase inhibitor PP2. Although EGF stimulation of Y265 phosphorylation was observed in P-UAEC-adEGFR and was reversed by PP2, there was no recovery of Ca²⁺ bursting. This contrasts with the inhibitory effects of VEGF in parental P-UAEC, which were reversed by either U0126 or PP2. These data suggest (Fig. 9) that while VEGF downregulation of Ca²⁺ bursting is mediated primarily through a single Src-dependent ERK pathway (Fig. 9A), the inhibitory action of EGF is mediated primarily through a single Src-*independent* ERK pathway. Secondly, the previously observed inhibitory effect of VEGF treatment on Ca²⁺ bursting is lost in P-UAEC-adEGFR, suggesting competition or redistribution of shared intermediate signaling molecules. We tentatively propose that the coupling of exogenous EGFR to the key effector molecules needed to achieve Cx43 phosphorylation (adapter proteins and intermediate kinases) necessitates the *decoupling* of VEGFR-2 to those same molecules (Fig. 9B) because the overexpression of EGFR in P-UAEC results in receptor localization in membrane regions where it is normally absent and so can access to pools of signaling molecules that it would otherwise be denied, and thereby sequestering these effectors from VEGFR2. The constitutive autophosphorylation of the receptor alters the C-terminal tail of EGFR to be 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 PLC γ . Despite the apparent negative feedback loop that resists the continuous activation of MEK and ERK in most cells, upstream effector molecules for the MAPK pathway are still likely to be “occupied” in interaction with the C-terminus of EGFR for much of their time at the membrane, reducing the availability of these molecules for other receptors if they are rate limiting in quantity. The loss of VEGF-mediated downregulation of Ca²⁺ bursting also suggests that constitutively active EGFR has a greater *affinity* for at least some key effector molecules of the ERK1/2 pathway than VEGFR-2. If this is indeed the case, it follows that maintaining low endogenous EGFR expression in P-UAEC *in vivo* may be *necessary* for VEGFR-2 to control ERK-mediated regulation of GJC, a necessary step to initiating angiogenesis in endothelial cells. This result also supports our proposal that VEGFR-2 is sufficient to mediate the inhibitory action of VEGF on Cx43 GJC, as previously suggested by the findings of Grummer et al., 2009 and Boeldt et al., 2015, which

found no evidence to suggest that the activity of VEGFR1 homodimers plays a significant role in the inhibitory action of VEGF on GJC. While a role for VEGFR-1/VEGFR-2 heterodimers in the regulation of GJC remains a possibility, it is unlikely to be a significant factor in mediating the acute Ca²⁺ response to ATP.

Finally, there are several additional physiologic implications of the low endogenous level of EGFR normally observed in P-UAEC, and physiologic consequences of overexpression above the physiologic norm. First, in the absence of ligand, endothelial cells may generally be able to contain overactivity of kinase pathways associated with Cx43 phosphorylation and keep GJC functional inhibition in check, thereby conferring resistance to the tumorigenic effects of aberrant EGFR signaling, unlike many cell types that are susceptible to EGFR-associated tumors. Indeed, the inability of EGFR overexpression to induce constitutive basal activation of Src and ERK pathways in P-UAEC could explain the low frequency of endothelial cell cancers (Jemal et al., 2005; Coindre et al., 2001).

Despite the lack of effect from constitutive ligand-independent EGFR activity, our data in P-UAEC-adEGFR suggest that further stimulation with EGF ligand can achieve dysfunction, and at a physiologically relevant level. While dramatic changes in endothelial EGFR expression have neither been reported or nor indeed are likely in normal pregnancy, it is important to note that placental trophoblasts do express large amounts of EGFR and release many extracellular vesicles (EVs) into the maternal circulation (Whigham et al., 2019). Were these exosomes to contain significant numbers of EGFR, it is possible that endothelium might be impacted with changes in function similar to those we describe here. Extracellular vesicles are lipid spheres with plasma membrane elements reflecting the cell of origin (e.g. placental alkaline phosphatase [PLAP] on the surface of placental villous syncytiotrophoblasts). Cell-specific EVs seem to be abundant in human blood (~10⁵–10⁸/ml; actual numbers are not entirely clear because of method limitations) and seem to play a role in altering cell-to-cell communication (Tkach and Thery 2016). In our experience, commonly employed exosome isolation methods like differential ultracentrifugation and affinity capture cannot provide the specificity necessary for accurate cell-and size-specific analysis of *placenta*-derived PLAP+/EGFR+ populations (Morgan 2018). To date it has been shown placental (PLAP+) exosome counts per milliliter of blood increase with preeclampsia (Knight et al., 1998; Salomon et al., 2017). Women who later develop preeclampsia seem to have more total exosomes and more PLAP+ EVs/ml of blood. We add to this body of literature by showing here for the first time that multiplex nanoscale flow cytometric imaging of first trimester plasma reveals a population of PLAP/EGFR positive EVs with greater variance in counts/volume than PLAP+ events alone. The implication is that floating villous syncytiotrophoblasts release exosomes and microvesicles that contain EGFR on their surface, which could potentially impact maternal endothelial function by delivery of functional EGFR. The limitation of this analysis was the absence of (1) pregnancy outcome data and (2) the absence of FACS sorted EVs for further validation studies.

Although PE characterized by endothelial dysfunction is not typically associated with elevated EGF, it is important to recognize that EGFR spontaneously elevated in endothelium could still be transactivated *in vivo* over the duration of pregnancy by circulating cytokines in the maternal vasculature, which are known to be significantly elevated in preeclampsia

(Reviewed in LaMarca et al., 2007; Lau et al., 2013). TNF α , IL-8, IL-1 α , IL-1 β , and IFN γ have all been reported to transactivate EGFR (Argast et al., 2004; Chen et al., 2004; Itoh et al., 2005; Tanida et al., 2004). IL-8, for example, has been shown to induce phosphorylations of a wide variety of signaling molecules via EGFR transactivation, including not only Src and ERK1/2, but also FAK, Jnk, p38-MAPK, STAT1 and STAT3. Transactivation was confirmed by pharmacological inhibition of IL-8 or EGFR, which abolished these phosphorylations (Kyriakakis et al., 2011). IL-8 can also transactivate EGFR in microvascular endothelium (Schraufstatter, 2003), suggesting that the presence of EGFR ligands may not be necessary for the receptor to mediate Cx43 phosphorylation in vivo. Accumulating data in rodent models suggest that aberrant EGFR signaling may also play a role in the development and/or maintenance of general hypertensive diseases (Florian and Watts, 1999; Sambhi et al., 1992; Kagiya et al. 2002, 2003; Kim et al., 2006; Ying and Sanders, 2005). More recently, the EGFR gene ranked highest in a mouse genome-wide association study to identify genes associated with susceptibility to high fat diet-induced pulmonary hypertension (Kelly et al., 2017). Our data suggest that EGFR in an abnormally elevated state could render the endothelium more susceptible to dysfunction triggered by naturally occurring EGFR ligands or transactivation, and in this context may contribute to hypertension. While excessive EGFR activation may be a rare event in the total PE population, our data suggest that the outcomes associated with it would be among the most extreme. To that end, our finding that exosomes of placental origin also stain positive for EGFR raises the very real possibility that trophoblasts shedding exosomes could directly impact endothelial function. Further studies in both exosome biology and endothelial cell changes in PE subjects will be needed to determine if indeed this is the case.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This work has been supported in part by NIH awards HD038843 (IMB) and HD089861 (TKM). This paper reports partial fulfillment of the requirements for LC towards a PhD in the University of Wisconsin Endocrinology and Reproductive Physiology Training Program. LC was also supported by T32 predoctoral training award (T32 HD041921).

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Highlights:

We have previously shown that VEGF can promote endothelial dysfunction by Src and Erk kinase-mediated phosphorylation and closure of Cx43 channels.

The role of VEGFR2 in this response is clear, but the role of VEGFR1 in this response is unclear.

Addition of EGFR can make endothelium newly responsive to EGF and fully reproduce Cx43 phosphorylation and closure.

Addition of EGFR also blocks VEGFR coupling to Cx43 phosphorylation and closure.

Physiologic relevance to preeclampsia is supported by placental exosomes being EGFR positive.

Figure 1. Expression and Cx43-related activity of endogenous EGFR in P-UAEC.

(A) P-UAEC were labeled with one of two primary antibodies--mAb528, an antibody with an affinity for the receptor's ligand binding site equal to EGF, or non-selective IgG control--and a phycoerythrin-tagged secondary antibody. The samples were then subjected to analysis using the FACSCalibur flow cytometer. The geometric mean of fluorescence intensity was quantified for 10,000 cells in each group. Surface expression of EGFR was detected in only 22% of cells. (n = 2) (B) EGF treatment does not inhibit the pregnancy-adapted Ca²⁺ burst function in P-UAEC. Cells were pre-loaded with the [Ca²⁺]_i-sensitive dye Fura-2, stimulated with ATP (100 μ M) for 30 min, washed with fresh buffer and allowed to sit for 30 min. Cells were then treated with VEGF (10 ng/mL), EGF (10 ng/mL), or vehicle control for 30 min, followed by re-stimulation with ATP for 30 min. Data was collected from cells showing 3 or more ATP stimulated Ca²⁺ bursts prior to growth factor treatment. Columns represent percent of initial mean burst number $\pm$ SE. Treatment with VEGF inhibited the number of Ca²⁺ bursts significantly below the control level (*** P < 0.0001), but EGF did not. Statistics were performed on raw data from individual P-UAEC observed in 5–22 dishes: control (n = 342), EGF (n = 226), or VEGF-165 (n = 778). Comparisons of post-treatment groups vs. control group were analyzed by Wilcoxon rank sum test. (C)

Phosphorylations of Cx43 Y265 or S279/282 are not significantly elevated in P-UAEC following 30-minute EGF treatment. Western blot analysis of P-UAEC lysates showed that phosphorylation of the Cx43 inhibitory site Y265 was increased 1.5-fold by 30-minute treatment with VEGF ($P = 0.029$), but not by EGF treatment. Phosphorylation of the Cx43 inhibitory site S279/282 showed no significant change following 30-minute treatment with VEGF or EGF. This is contrary to prior research published by the Bird laboratory, which found that 30-minute VEGF treatment significantly elevated pS279/282 1.4-fold (Boeldt et al. 2015). Black lines in boxes indicate median values. Statistical significance was determined by ANOVA and Tukey's HSD post-hoc test. ($n = 6$).

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Figure 2. EGFR expression as a function of adenoviral multiplicity of infection (MOI).

(A) Western blot analysis was performed on lysates of P-UAEC adenovirally transduced with the wild-type EGFR gene at multiplicities of infection (MOIs) of 0, 10, 100, and 1000 using the EGFR(1005) antibody (Santa Cruz), which is selective for the C-terminal tail of EGFR, and normalized using a Hsp90 control. Expression of EGFR in P-UAEC is undetectable in parental cells without excessive overexposure, but became apparent at an MOI of 100 and was robust at an MOI of 1000. Quantification of bands indicated that P-UAEC in the MOI 1000 group expressed EGFR at 110-fold of control (histogram not shown, $n = 8$), but this fold increase is likely not precise/is overestimated since cells transduced at less than an MOI of 100 are at or below the threshold of detection sensitivity.

(B) P-UAEC were labeled with the EGFR-selective mAb528 primary antibody or non-selective IgG control and phycoerythrin-tagged secondary antibody. Cells were then subjected to analysis using the FACSCalibur flow cytometer as described in the Methods section. Flow cytometric analysis showed that the percentage of P-UAEC that expressed EGFR on the cell surface increased with higher MOI. Expression of exogenous EGFR was far from uniform at lower MOI, and varied widely from cell to cell in each sample. At an MOI of 3200, 100% of P-UAEC expressed detectable EGFR. Exposure to adenovirus had no negative effect on cell viability (data not shown). ($n = 2$).

Figure 3. Overexpressed EGFR in P-UAEC is constitutively autophosphorylated at an adenoviral MOI of 1000.

Lysates of parental P-UAEC and P-UAEC adenovirally transduced with the wild-type EGFR gene at varying multiplicities of infection (MOI) were immunoblotted with a pY1173-selective antibody and normalized using a Hsp90 control. (A) No significant basal or ligand-responsive autophosphorylation of EGFR was detected in parental P-UAEC or the MOI 10 cells. Adenoviral transduction of the EGFR gene in P-UAEC at an MOI of 100 resulted in visible autophosphorylation in response to 30-minute EGF treatment (10 ng/mL) but did not display constitutive autophosphorylation. (B) Clear and consistent constitutive autophosphorylation of Y1173 was readily apparent in the MOI 1000 sample group (*** $P = 0.001$). Black lines in boxes indicate median values. Statistical significance was determined by ANOVA and Tukey's HSD post-hoc test. ($n = 4$).

Figure 4. Basal and EGF-induced levels of pERK1 and pERK2 in parental P-UAEC and P-UAEC-adEGFR.

(A) Western blot analysis revealed no significant differences in basal ERK phosphorylation between cells adenovirally transduced with EGFR at MOIs of 10, 100, or 1000; however, a trend of increased MOI inducing greater pERK1/2 was observed. Cells transduced at an MOI of 1000 exhibited the greatest increases of mean basal ERK1 and ERK2 phosphorylations at 2.1- and 1.9-fold, respectively, above the levels observed in parental P-UAEC, but these elevations were not found to be significant by ANOVA due to the high variance between trials. (n = 9) (B) Western blot analysis also revealed no significant differences in basal phosphorylations of Cx43 Y265 or S279/282 between cells adenovirally transduced with EGFR at MOIs of 10, 100, or 1000. (C) Robust phosphorylations of ERK1 (P = 0.001) and ERK2 (P = 0.001) were observed in P-UAEC-adEGFR following 30-minute treatment with EGF (10 ng/mL). However, administration of VEGF at 30 minutes showed no change. Scale on vertical axis represents fold of basal pERK1 (left) or pERK2 (right) in P-UAEC-adEGFR. (n = 7) All values were normalized to Hsp90 protein level. Black lines in

boxes indicate median values. Statistical significance was determined by ANOVA and Tukey's HSD post-hoc test.

Figure 5. Basal and growth factor-induced levels of Akt pS473 in parental P-UAEC and P-UAEC-adEGFR.

(A) Western blot analysis of Parental P-UAEC and P-UAEC-adEGFR lysates found the mean elevation of basal phosphorylation of Akt S473 to be over 16-fold ($P = 0.021$), suggesting that constitutive activation of the PI3K/Akt pathway is not negatively regulated as the MEK/ERK pathway appears to be. Data collected from the results of 7 independent experiments. Statistical significance was determined by Welch's t-test. (B) Parental P-UAEC and P-UAEC-adEGFR were treated with vehicle control, EGF (10 ng/mL), or VEGF (10 ng/mL) for 30 min and subjected to western blot analysis. Neither EGF nor VEGF induced significant phosphorylation of Akt S473 in parental P-UAEC. In P-UAEC-adEGFR, however, treatment with EGF raised S473 phosphorylation 2.6-fold ($*** P = 0.003$) over the already elevated basal level (equivalent to 44-fold of basal level in parental P-UAEC). VEGF treatment had no effect on the level of Akt pS473 in P-UAEC-adEGFR. All values were normalized to Hsp90 protein level. Black lines in boxes indicate median values. Statistical significance was determined by ANOVA and Tukey's HSD post-hoc test. ($n = 7$).

Figure 6. Ligand-dependent activation of EGFR in P-UAEC-adEGFR downregulates Ca²⁺ bursting to a level consistent with VEGF in parental P-UAEC.

(A) Constitutive activity of EGFR in P-UAEC-adEGFR does not downregulate the calcium burst response to ATP. By aggregating the data from the initial ATP-stimulated response of every calcium imaging trial, we were able to compare the mean number of Ca²⁺ bursts exhibited by parental P-UAEC (1346 cells) vs. P-UAEC-adEGFR (1147 cells) prior to any administration of kinase inhibiting drugs or growth factors. Parental P-UAEC and P-UAEC-adEGFR pre-loaded with the [Ca²⁺]_i-sensitive dye Fura-2 were stimulated with ATP (100 μ M) and the number of Ca²⁺ bursts occurring in 30 min were counted for each selected cell. Data was collected only from cells showing 3 or more ATP stimulated Ca²⁺ bursts. Contrary to our hypothesis, the mean number of Ca²⁺ bursts per cell was not reduced in P-UAEC-adEGFR, but 4% greater (** P = 0.005) than the mean number of bursts in parental P-UAEC. P-value was obtained by unpaired t-test. (B) EGF inhibits the pregnancy adapted Ca²⁺ burst function in P-UAEC-adEGFR to a level consistent with VEGF-induced inhibition in parental P-UAEC. Fura-2-loaded parental P-UAEC or P-UAEC-adEGFR were stimulated with ATP (100 μ M) for 30 min, then washed with fresh buffer and allowed to sit for 30 min. Cells were subsequently treated with VEGF (10 ng/mL), EGF (10 ng/mL), or vehicle control for 30 min, then re-stimulated with ATP for 30 min. Columns represent percent of initial mean burst number $\pm$ SE. In parental P-UAEC, treatment with VEGF inhibited the number of Ca²⁺ bursts significantly below the control level, but EGF did not. However, in P-UAEC-adEGFR the results are reversed. EGF induced significant inhibition, whereas VEGF had no effect on Ca²⁺ bursting. Data was collected from individual P-UAEC

observed in 5–22 dishes: control (n = 342), EGF (n = 226), or VEGF-165 (n = 778) in parental P-UAEC; EGF (n = 362) or VEGF-165 (n = 313) in P-UAEC-adEGFR. Comparisons of post-treatment groups vs. control group were analyzed by Wilcoxon rank-sum test (***) $P < 0.0001$). (C) Consistent with the Ca^{2+} burst data, western blot analysis revealed that EGF treatment of P-UAEC-adEGFR induced phosphorylations of Cx43 Y265 (1.4-fold; * $P = 0.044$) and S279/282 (2.9-fold; *** $P = 0.001$), but neither site was significantly altered by VEGF. All values were normalized to Hsp90 protein level. Black lines in boxes indicate median values. Statistical significance was determined by ANOVA and Tukey's HSD post-hoc test. (n = 6).

Figure 7. Effects of pharmacological inhibition of Src or MEK on EGF-mediated downregulation of Ca²⁺ bursting in P-UAEC-adEGFR.

(A) Pretreatment for 30 min with the MEK-selective inhibitor U0126 (10 μ M) prior to EGF administration had no effect on Cx43 Y265 phosphorylation (left), which is known to be mediated by Src, but the SFK-selective inhibitor PP2 (10 μ M) kept Cx43 Y265 phosphorylation at a level not significantly different from basal pY265. Inversely, 30-minute pretreatment with PP2 had no effect on S279/282, while U0126 prevented EGF-induced elevation of pS279/282 (right). All values were normalized to Hsp90 protein level. Black lines in boxes indicate median values. Statistical significance was determined by ANOVA and Tukey's HSD post-hoc test. (B) ATP-stimulated Ca²⁺ bursting was fully "rescued" from EGF inhibition by blocking the MEK/ERK signaling pathway, but not by blocking c-Src. Pretreatment with U0126 prior to EGF treatment completely protected P-UAEC-adEGFR from EGF inhibition of Ca²⁺ bursting. However, pretreatment for 30 min with PP2 prior to EGF treatment did not protect against loss of Ca²⁺ bursts. These data suggest that EGF inhibition of CCE is mediated via the MAPK pathway, but not the Src pathway. Differences between treatments were compared by Wilcoxon rank-sum test (***) $P < 0.0005$. (Control, $n = 342$; EGF, $n = 362$; U0126 + EGF, $n = 199$; PP2 + EGF, $n = 225$).

Figure 8. Nanoscale flow cytometric analysis of placental exosomes in first trimester plasma. Platelet poor plasma diluted in 0.1µm filtered PBS buffer stained for (A) CD63 tetraspanin, (B) placental alkaline phosphatase, (C) epidermal growth factor, and (D) PLAP/EGFR co-labeling of PLAP+ and EGFR+ exosomes. (E) Quantitative analysis comparing extracellular vesicle (EV) counts in batch of 36 first trimester cases compared with 4 male negative controls and stained PBS sample dilution buffer. Note that 200nm bead counts/ul of plasma tested are the same between groups (same volumes tested). Means were compared by ANOVA with post-hoc correction (** P<0.01).

Figure 9. Putative Explanation for the Failure of VEGF-A165 to Inhibit Ca²⁺ Bursting in P-UAEC-adEGFR.

(A) Prior data from Boeldt et al. (2015) suggest that VEGF administration (10 ng/mL) triggers the formation of activated VEGFR-2 homodimers that initiate three relevant signaling cascades. The first is Src-independent activation of MEK/ERK, which in turn mediates inhibitory phosphorylations of Cx43 proteins at S279/282. The second is Src-dependent activation of MEK/ERK in an unknown manner, possibly by direct interaction of Src with Raf-1 (Chao et al. 1997), followed by ERK-mediated phosphorylation of Cx43 S279/282. The third is direct Src-mediated phosphorylation of Cx43 Y265. Interestingly, pharmacological inhibition of either Src (PP2, 10 μ M) or MEK (U0126, 10 μ M) protects the Ca²⁺ burst response from VEGF-induced downregulation. Evidence provided by EGF treatment of P-UAEC-adEGFR shows that ERK-mediated phosphorylation of Cx43

S279/282 is required for downregulation of the ATP-induced Ca²⁺ burst response, but Src-mediated phosphorylation of Cx43 Y265 is not (Fig. 7B). These data suggest that PP2 inhibition of Src protects Ca²⁺ bursting from the effects of VEGF specifically because it prevents Src activation of ERK1/2, thereby revealing pathway #2 (bold arrows) to be the critical mediator of acute VEGF-induced downregulation of GJC. (B) Although constitutive ligand-independent activity failed to induce significant elevations of basal ERK1/2 or inhibitory phosphorylations of Cx43, it appears to have a profound effect 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 P-UAEC-adEGFR as it did in parental P-UAEC (Fig. 6B). 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 might 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 PLC γ . Despite the apparent negative feedback loop that resists the perpetual activation of MEK and ERK, upstream effector molecules are still likely to be “locked” in interaction with the C-terminus of EGFR for much of their time at the membrane, thus reducing their availability for other receptors. The loss of VEGF-mediated effects on Cx43 in P-UAEC-adEGFR suggests that EGFR may have a higher affinity for these effectors than VEGFR-2.

Table 1:
Number of receptors per cell as a function of the geometric mean of fluorescence intensity determined by FACS.

By comparing to the known EGFR expression level in A431 cells ($\sim 2.6 \times 10^6$ receptors/cell), parental P-UAEC were found to express ~ 7000 EGFR/cell. Adenoviral transduction at MOIs of 1000 and 3200 increased expression 26-fold and 75-fold, respectively.

MOI	Mean fluorescence intensity	EGF receptors per cell	Fold of control
0	13.3	7000	1.0
10	17.0	9100	1.3
100	61.5	33000	4.7
320	123	66000	9.4
1000	337	180000	26
3200	985	530000	75
A431	4860	2600000	371