MOLECULAR REGULATORS OF EMBRYO IMPLANTATION AND PREGNANCY

Brooke Campbell Matson

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Approved by:

Kathleen Caron

Keith Burridge

Richard Cheney

Robert Tarran

Steven Young

ABSTRACT

Brooke Campbell Matson: Molecular Regulators of Embryo Implantation and Pregnancy
(Under the direction of Kathleen Caron)

Embryo implantation is one of the first steps in the establishment of pregnancy and sets the stage for proper embryonic and placental development. We are beginning to appreciate the complexity of implantation and its demand for synchrony between embryo and endometrium. Over the years, many groups have identified and studied both embryonic and endometrial factors affecting implantation, but there is still much to learn. Here, we focus on the endocrine peptide adrenomedullin (*Adm*, AM) and its association with female reproductive physiology and disease in both mice and humans.

In Part I, we elaborate on the subfertility phenotype of *Adm* heterozygous female mice by testing whether AM supplementation before implantation can improve fertility and pregnancy outcomes. We find that administration of AM directly to the mouse uterus before blastocyst transfer enhances implantation success and inter-embryonic spacing. Intra-uterine delivery of AM also confers morphological and cellular changes to the uterine epithelium and stroma that are associated with endometrial receptivity and believed to be beneficial for implantation. We also test mid-regional pro-adrenomedullin (MR-proADM), a stable surrogate for AM peptide, as a biomarker for endometriosis, which is comorbid with infertility. Our studies identify novel mechanisms of action of AM in the endometrium in support of implantation and the establishment of pregnancy, providing a foundational basis for the use of AM as a clinical therapeutic for infertility.

In Part II, we address the role of AM in the maintenance of a healthy pregnancy by expanding upon previous studies in mouse models with clinical samples from women with severe preeclampsia, a placental vascular disorder characterized by hypertension and

proteinuria. Because *Adm*$^{-/-}$ placentas exhibit a preeclampsia-like phenotype, we test the hypothesis that MR-proADM concentrations are decreased in plasma from women with severe preeclampsia. Indeed, we find that MR-proADM levels are blunted in women with severe preeclampsia and that MR-proADM is similarly effective as other established biomarkers of preeclampsia at distinguishing between cases and controls. Altogether, our studies in both mice and humans point to diagnostic and therapeutic applications for AM in the context of female reproductive disease.

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LIST OF ABBREVIATIONS

Adm	adrenomedullin gene
AHR	aryl hydrocarbon receptor
AM	adrenomedullin protein
AM2	adrenomedullin 2 protein
ART	assisted reproductive technology
BSA	bovine serum albumin
CFH	complement factor H
CFTR	cystic fibrosis transmembrane conductance regulator
CGRP	calcitonin gene-related peptide
ChIP	chromatin immunoprecipitation
Cldn1, CLDN1	claudin 1 (*gene*, protein)
CLR	calcitonin receptor-like receptor
cKO	conditional knockout
CM	conditioned media
Cx43	connexin 43 protein
DBA	*Dolichos biflorus* agglutinin
e	embryonic day
ERA	endometrial receptivity array
FGR	fetal growth restriction
gd	gestational day
Gja1	connexin 43 gene
hESC	human endometrial stromal cell
HIF1A	hypoxia inducible factor 1A
HLA	human leukocyte antigen

HUVEC	human umbilical vein endothelial cell
IFN-γ	interferon-γ
IL-6	interleukin-6
IL-15	interleukin-15
I_{SC}	short-circuit current
IUGR	intrauterine growth restriction
IUI	intrauterine insemination
IVF	*in vitro* fertilization
KIR	killer cell Ig-like receptor
LIF	leukemia inhibitory factor
MHC	major histocompatibility complex
MLAp	mesometrial lymphoid aggregate of pregnancy
MMP	matrix metalloproteinase
MR-proADM	mid-regional pro-adrenomedullin
pbNK	peripheral blood natural killer
PDZ	primary decidual zone
PlGF	placental growth factor
pSTAT3	phosphorylated signal transducer and activator of transcription 3
qRT-PCR	quantitative real-time polymerase chain reaction
RAMP	receptor activity modifying protein
RIF	recurrent implantation failure
ROC	receiver operating characteristic
SEM	scanning electron microscopy
sEng	soluble endoglin
STAT3	signal transducer and activator of transcription 3
STRE	STAT3 response element

TER	transepithelial resistance
TGC	trophoblast giant cell
uNK	uterine natural killer

PART I – ESTABLISHMENT OF PREGNANCY

Chapter 1: Introduction – Embryo Implantation and Adrenomedullin

Embryo Implantation and Endometrial Receptivity

Proper blastocyst implantation is vital for the establishment of pregnancy and hinges upon endometrial receptivity, among many other factors.[1,2] Aberrant endometrial receptivity is an enigmatic cause of infertility that even "last-line" assisted reproductive technology (ART), *in vitro* fertilization (IVF), cannot overcome.[3-5] While endometrial "sub-receptivity" may permit implantation, it may also program adverse maternal and fetal outcomes that present later in pregnancy.[6,7] Unfortunately, very little is understood about the molecular mechanisms underlying endometrial receptivity and blastocyst implantation. Therefore, many cases of infertility are unexplained and unresolved despite the use of ART.[8] Likewise, the etiologies of many pregnancy complications are incompletely understood. Advances in our understanding of the signaling pathways underlying endometrial receptivity will therefore shed light on potential therapeutics for infertility and complications of pregnancy, or conversely, on innovative contraceptive methods.

Receptivity is achieved by a complex dialogue between the blastocyst and the epithelial and stromal compartments of the uterus during the peri-implantation period, which occurs between embryonic day (e) 0 to e4.5 and shortly thereafter in mice.[9-11] At e4.5, the blastocyst attaches to the uterine luminal epithelium, and the trophectoderm layer – the

outer layer of the blastocyst surrounding the inner cell mass – begins to invade the endometrium, the inner lining of the uterus. Concurrently, uterine stromal cells adjacent to the implantation site undergo a differentiation process termed decidualization to generate decidual cells that provide support to the embryo prior to the development of the placenta.[12] Decidual cells immediately surrounding the embryo organize into a transient, avascular zone termed the primary decidual zone (PDZ), which is believed to form a highly selective barrier around the embryo to prevent rejection by the maternal immune system.[13]

A salient signaling pathway for these processes is the leukemia inhibitory factor-signal transducer and activator of transcription 3 (LIF-STAT3) axis.[14,15] The LIF receptor, via Janus kinase (JAK), phosphorylates STAT3, stimulating its translocation into the nucleus and the transcription of effector genes.[16] Female mice with a global *Lif* deletion or with a pan-uterine or uterine epithelial-specific *Stat3* deletion (*Stat3*$^{d/d}$) are all infertile due to defects in implantation and decidualization.[17-19] More specifically, *Stat3*$^{d/d}$ females demonstrate impaired uterine epithelial remodeling during the peri-implantation period due in part to aberrant expression and localization of junctional proteins.[17] Two downstream targets of STAT3 during the pre-implantation period have been identified in the uterus – early growth response 1 (*Egr1*) and epidermal growth factor receptor (EGFR) signaling – but others remain to be discovered.[17,20]

Adrenomedullin and the Establishment of Pregnancy[1]

The peptide hormone adrenomedullin (*Adm,* AM) is a candidate gene target of *Stat3*.[21-23] Originally isolated from pheochromocytoma extracts,[24] AM is a vasodilatory, angiogenic, and anti-inflammatory protein with demonstrated roles in cardiac and lymphatic vascular development and tumor biology.[25,26] AM belongs to the calcitonin/calcitonin gene-

[1]Reprinted in part with permission from: Matson BC & Caron KM. Adrenomedullin and endocrine control of immune cells during pregnancy. *Cell Mol Immunol*. 2014;11:456-459.

related peptide (CGRP) family, which binds various combinations of G-coupled protein receptors (GPCRs) and their associated receptor activity modifying proteins (RAMPs). The canonical receptor for AM is calcitonin receptor-like receptor (*Calcrl*, CLR) when associated with either RAMP2 or 3.[27] Estrogen and hypoxia, which are elevated throughout pregnancy, dramatically upregulate either *Adm* or *Calcrl* gene expression in several human and rodent female reproductive tissues including the uterus, ovary, and placenta, thus underscoring the significance of AM signaling in female-specific reproductive physiology.[28-33]

At the organismal level, plasma concentrations of AM are elevated two-to-three-fold above baseline levels in many disease states, such as cardiovascular, hepatic, renal, and pulmonary disease, but interestingly, the largest increase in plasma AM levels occurs during a healthy pregnancy.[34] Whether this physiological elevation occurs during complications of pregnancy remains uncertain.[35] However, polymorphisms in the human *Adm* gene are associated with preeclampsia,[36] and administration of an AM antagonist to pregnant rats causes placental and fetal pathologies.[37] A newly developed assay to detect a proteolytically cleaved precursor of active AM, mid-regional pro-adrenomedullin (MR-proADM), provides an alternative way to quantitate AM in humans and is currently being investigated as a biomarker of cardiovascular disease, pneumonia, and sepsis.[38] While data on changes in AM levels in complications of pregnancy have been inconsistent, MR-proADM provides hope that consensus about changes in plasma AM levels in reproductive diseases can be achieved and may potentially be used as a surrogate for the prognostic determination of pregnancy complications.[39]

At the cellular level, many studies have described *Adm* expression in tissues derived from both the mother and the fetus: ovary, uterus, placenta, and fetal membranes.[40-46] For example, just prior to implantation in mice, *Adm* is highly expressed in the trophectoderm cells of the early blastocyst and the luminal epithelial cells of the uterine lining.[42,47,48] Shortly after implantation, and during the rapid expansion of the murine decidua, *Adm* expression is

strongly centered within the primary decidual zone (PDZ), a 3-5 cell-layer thick region surrounding the recently implanted embryo which serves as a temporary and physical barrier to immunological attack.[48] However, *Adm* expression is most enriched in mouse trophoblast giant cells (TGCs) throughout pregnancy, with approximately 30-fold higher levels in differentiated TGCs compared to undifferentiated precursors.[48] Because TGCs are active players in the processes of implantation, decidualization, and placentation, this robust expression of *Adm* from these fetal cells implicates AM in many stages of pregnancy.[49] Moreover, TGCs of $Adm^{-/-}$ placentas undergo apoptosis, further suggesting that AM is critical for the survival of these cells that are central to the maintenance of a healthy pregnancy.[50]

During the generation of gene-targeted $Adm^{-/-}$ mice, which are embryonic lethal by e14.5,[51] it was observed that $Adm^{+/-}$ females had smaller litters than their wild type counterparts, prompting questions about the fertility of $Adm^{+/-}$ dams. Subsequently, it was demonstrated that wild type expression levels of *Adm* are important for endometrial receptivity in mice during the peri-implantation period, specifically via the promotion of pinopode formation – a proxy for endometrial receptivity – in the uterine luminal epithelium.[47] Healthy implantation, however, is likely determined by factors beyond uterine luminal epithelium, such as appropriate tempering of maternal immunity. Based on the interaction of AM with its anti-inflammatory binding partner, complement factor H, one could speculate that AM is also important for preventing an immune attack on the embryo during the peri-implantation period.[47]

Embryos that are able to implant in $Adm^{+/-}$ uteri do so unevenly both within and between uterine horns.[48] It is possible that this $Adm^{+/-}$ implantation phenotype is due in part to changes in ciliary beat frequency in the oviduct.[52,53] Embryos of $Adm^{+/-}$ dams are also more likely to die or demonstrate abnormalities symptomatic of poor placental perfusion during the development of the placenta between e9.5 and e12.5.[48] Furthermore, placental morphologies observed in embryos developing within $Adm^{+/-}$ uteri exhibit aberrant invasion

of *Adm*-expressing TGCs into the decidua.[48] Collectively, these studies in *Adm*$^{+/-}$ female mice demonstrate that the expression and dosage of maternal AM is a critical determinant of normal endometrial receptivity and proper implantation. These studies also suggest that administration of AM directly into the uterus prior to implantation or early pregnancy may improve fertility and pregnancy outcomes. In this next chapter, we test this hypothesis and shed additional light on the pathophysiology of the *Adm*$^{+/-}$ subfertility phenotype.

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Chapter 2: Adrenomedullin Improves Fertility and Promotes Pinopodes and Cell Junctions in the Peri-Implantation Endometrium[1]

Overview

Implantation is a complex event demanding contributions from both embryo and endometrium. Despite advances in assisted reproduction, endometrial receptivity defects persist as a barrier to successful implantation in women with infertility. We previously demonstrated that maternal haploinsufficiency for the endocrine peptide adrenomedullin (AM) in mice confers a subfertility phenotype characterized by defective endometrial receptivity and sparse epithelial pinopode coverage. The strong link between AM and implantation suggested the compelling hypothesis that administration of AM prior to implantation may improve fertility, protect against complications, and ultimately lead to better maternal and fetal outcomes. Here, we demonstrate that intrauterine delivery of AM prior to blastocyst transfer improves the embryo implantation rate and spacing within the uterus. We then use genetic decrease-of-function and pharmacologic gain-of-function mouse models to identify potential mechanisms by which AM confers enhanced implantation success. In epithelium, we find that AM accelerates the kinetics of pinopode formation and water transport, and in stroma, that AM promotes connexin 43 expression, gap junction communication, and barrier integrity of the primary decidual zone. Ultimately, our findings advance our understanding of the contributions of AM to endometrial receptivity and suggest

[1]Adapted with permission from: Matson BC, Pierce SL, Espenschied ST, Holle E, Sweatt IH, Davis ES, Tarran R, Young SL, Kohout TA, van Duin M & Caron KM. Adrenomedullin improves fertility and promotes pinopodes and cell junctions in the peri-implantation endometrium. *Biol Reprod*. 2017;97(3): 466-477.

potential broad use for AM as therapy to encourage healthy embryo implantation, for example in combination with in vitro fertilization.

Introduction

Blastocyst or embryo implantation is essential for the establishment of pregnancy and occurs during the window of implantation between embryonic days (e) 3.5-4.5 in mice and between days 6-12 post-fertilization in humans.[1] While embryo implantation takes place during the very early days of pregnancy, errors occurring during this process can program clinical complications of pregnancy that present later in gestation, leading to adverse maternal and fetal outcomes.[2] A complex dialogue between the embryo and the endometrium coordinates this delicate and precisely timed event, offering two angles from which to approach the clinical problem of infertility, which affects millions of women and families across the world.[3,4] Modern assisted reproductive technology (ART) can address embryonic defects preventing implantation and the establishment of pregnancy by screening embryos for aneuploidy. However, in women, only about half of the euploid blastocysts transferred to the endometrial cavity in an optimally prepared uterus result in a successful pregnancy, underlining the inability of current medical techniques to alter endometrial receptivity and the need for development of improved therapies.[5]

Both the embryo and the uterus express cell surface molecules that enable their physical interaction, facilitating apposition and attachment of the blastocyst to the uterine luminal epithelium. Following closure of the epithelium around the embryo, embryonic trophoblast cells breach this layer by entosing living epithelial cells.[6] While the luminal epithelium is the first cell type that comes into direct contact with the embryo, uterine stromal cells also play a large role during the peri-implantation period. Stromal cells surrounding the implantation crypt undergo a progesterone-driven differentiation process termed decidualization that enable these cells to provide support to the embryo prior to the

development of a placenta.[7] A subpopulation of decidualized stromal cells adjacent to the embryo coordinate the formation of a transient, avascular region called the primary decidual zone (PDZ), which has been hypothesized to form a highly selective barrier that protects the embryo from immunological attack.[8] Despite advances in our understanding of these peri-implantation events in recent years, there is still much to be discovered about the molecular mechanisms underlying endometrial receptivity and implantation.

Previously, we and others established the peptide hormone adrenomedullin (*Adm* gene, AM protein) as an endocrine factor derived from both the mother and the fetus that is important for implantation, placentation, and the overall health of a pregnancy.[9,10] Notably, female mice heterozygous for *Adm* display a subfertility phenotype, demonstrating decreased pregnancy success and decreased epithelial pinopode coverage.[11,12] Litters of *Adm*$^{+/-}$ dams demonstrate irregular embryonic spacing and crowding *in utero* as well as fetal growth restriction and loss, yielding smaller litter sizes at weaning.[12] However, the cellular and molecular pathways downstream of AM peptide in the uterus remain to be elucidated.

During the peri-implantation period, *Adm* is spatiotemporally co-expressed with components of adherens junctions, tight junctions, and gap junctions in luminal epithelium and in decidualized stroma.[11,13-15] Notably, aberrant junctional protein expression and localization in these compartments can cause problems during implantation and decidualization with implications for fertility. For example, conditional deletion of several different transcription factors alters endometrial receptivity by interfering with expression of the tight junction protein claudin-1 (*Cldn1* gene, CLDN1 protein).[16-18] Furthermore, decreased connexin 43 (*Gja1* gene, Cx43 protein) function via a dominant loss-of-function mutation or administration of a pharmacological inhibitor interferes with early implantation events, specifically decidualization and early placental angiogenesis with consequences for fetal health.[19,20]

Given the spatiotemporal co-expression of *Adm* and cell junction proteins in the uterus during peri-implantation, AM may promote cell- and tissue-level organization by affecting junctional proteins. For example, in vitro studies on lymphatic endothelium previously demonstrated that AM induces organization of VE-cadherin and ZO-1.[21] We also found that AM promotes Cx43 mRNA and protein expression; Cx43 plasma membrane linearization; and gap junction coupling and intercellular communication, all in lymphatic endothelial cells.[22] By extension, this precedent for an AM effect on cell junctions may also apply to other cell types, which we evaluate in this current study. When taken together, the subfertility phenotype of $Adm^{+/-}$ dams and evidence for AM-cell junction interactions suggest the compelling hypothesis that AM promotes cell junction integrity in epithelial and stromal cells of the uterus, supporting the early embryo during an active time of complex tissue remodeling and thereby bolstering fertility. Here, we test this hypothesis and demonstrate that AM improves implantation success and spacing in mice and promotes cell junction organization in the peri-implantation uterine epithelium and stroma.

Materials and Methods

Animals

Mice with a deletion of the *Adm* gene were previously described and were maintained as a heterozygote colony on an isogenic 129S6/SvEv background.[23] Genotyping was performed using three primers – primer 1: 5′-CAGTGAGGAATGCTAGCCTC-3′; primer 2: 5′-GCTTCCTCTTGCAAAACCACA-3′; primer 3: 5′-TCGAGCTTCCAAGGAAGACCAGG-3′. Primers 1 and 3 amplify the wild type allele (1.8 kb), while primers 2 and 3 amplify the targeted allele (1.3 kb). All animal experiments were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Blastocyst transfer

Wild type CD1 female mice at least 8 weeks old (Charles River) were mated with vasectomized CD1 IGS males (Charles River) to generate pseudopregnant females. The morning of the vaginal plug was designated pseudopregnant day 0.5. On pseudopregnant day 2.5, e3.5 blastocysts were collected from superovulated C57BL/6 donor females (Envigo). Immediately prior to blastocyst transfer, 0.9% NaCl or 150 pmol AM (4.3 µl) (Phoenix Pharmaceuticals) was injected directly into each horn of the uterus of pseudopregnant females anesthetized with tribromoethanol (0.4 mg/g body weight). AM was co-injected with AM(24-50) (6.15 µl total) (Phoenix Pharmaceuticals) at a 20:1 AM(24-50):AM molar ratio or with complement factor H (CFH) (5.73 µl total) (R&D Systems) at a 3.3:1 CFH:AM weight ratio. Eight blastocysts were transferred into uterine horns treated with AM or AM + AM(24-50), and 16 blastocysts were transferred into uterine horns treated with AM + CFH. Recipient females were euthanized three days later after a tail vein injection of 0.1 mL 1% Evans blue dye (Sigma-Aldrich) in 0.9% NaCl. Embryo spacing was calculated in ImageJ (NIH) using the quantitation method depicted in Figure 2-1C.

In vivo barrier permeability assay

Wild type and *Adm*$^{+/-}$ females at least 8 weeks old were used for timed pregnancies. Visualization of the vaginal plug was considered e0.5. On e7.5, mice were anesthetized with tribromoethanol and injected retro-orbitally with 1 mg biotinylated bovine serum albumin (BSA). Two hours after injection, mice were euthanized, and implantation sites were dissected and fixed overnight in 4% paraformaldehyde at 4°C. Following fixation, implantation sites were cryoprotected in 30% sucrose and frozen in optimal cutting temperature (OCT) compound for sectioning. Tissue sections were stained with diaminobenzidine to evaluate the extent of BSA penetration toward the embryo.

Wet:dry weight

Day 2.5 pseudopregnant females were treated with a direct intrauterine injection of 0.9% NaCl or 150 pmol AM (4.3 µl) (Phoenix Pharmaceuticals). Thirty minutes later, animals were euthanized and dissected. A segment of uterine tissue was weighed at the time of dissection, incubated in a 60°C oven for 72 hours, and then re-weighed.

Optical projection tomography

After fixation in 4% paraformaldehyde, e5.0 implantation sites were transferred to PBS/0.1% Triton X-100 (PBST) and washed overnight at 4°C. Implantation sites were then boiled for 10 minutes in 10 mM citric acid/0.05% Tween 20 and allowed to cool to room temperature. Tissue was subsequently washed at room temperature for 5 minutes in PBST, 5 minutes in ddH_2O, 7 minutes in ice cold acetone, 5 minutes in ddH_2O, and 5 minutes in PBST. The tissue was then blocked overnight at 4°C in 5% normal donkey serum/1% BSA/1% DMSO in PBST. Samples were incubated at 4°C for one week in primary antibodies: Cx43 (1:200, Sigma-Aldrich) and FITC-conjugated IB4 lectin (1:100, Sigma-Aldrich) diluted in blocking solution. Samples were washed 7 x 15 minutes + 1 hour in 1% BSA/1% DMSO in PBST then incubated for 90 minutes in blocking solution. Secondary antibody (DyLight 594 donkey anti-rabbit, 1:100, Jackson ImmunoResearch) was diluted in blocking solution, and specimens were incubated for 3 days at 4°C. Tissues were then washed 8 x 15 minutes, transferred to PBST and washed overnight. Briefly, to prepare samples for OPT scanning, implantation sites were embedded in 1% low-melt agarose and dehydrated overnight in methanol at room temperature. Embedded specimens were rendered optically transparent by incubating them in benzyl alcohol and benzyl benzoate (BABB) for 48 hours before being scanned in a BiOPTonics 3001M Optical Projection Tomography Scanner equipped with Cy2 and Texas Red filter sets.

Scanning electron microscopy

Uterine horns of wild type CD1 females at least 8 weeks old (Charles River) were injected with 0.9% NaCl; 150 pmol AM; or 150 pmol AM + 3 nmol AM(24-50) on pseudopregnant day 2.5. At the time points specified in Figure 2-4, the animals were euthanized, and the uteri were dissected and cut open longitudinally. Uteri were submerged in a fixative containing 2% paraformaldehyde/2.5% glutaraldehyde/0.15 M sodium phosphate, pH 7.4 and stored at 4° overnight to several days before processing. After several washes in buffer, the samples were dehydrated through an ethanol dilution series (30%, 50%, 75%, 90%, 100%, and 100%) and subsequently dried using a Samdri-795 critical point dryer (Tousimis Research Corporation) with liquid carbon dioxide as the transition solvent. The tissue was then mounted onto aluminum scanning electron microscopy stubs with carbon adhesive tabs and was sputter coated with gold:palladium alloy (60:40) to a thickness of 20 nm using a Hummer X Sputter Coater (Anatech, Ltd.). Specimens were visualized with a Zeiss Supra 25 field emission scanning electron microscope (Carl Zeiss SMT) using an accelerating voltage of 5 kV. Quantitation of pinopode number, size, and percent area were performed in ImageJ (NIH). Images were batch processed with a macro that binarized, inverted, eroded, and then analyzed each image for particles greater than 0.2 μm in area and with a circularity between 0.3-1.00. Alternatively, pinopode number was manually determined using an ImageJ Cell Counter plugin.

Immunofluorescence

Paraffin-embedded tissues were sectioned, deparaffinized, and hydrated. Following antigen retrieval in 10 mM citric acid/0.05% Tween 20, pH 6.0, tissues were permeabilized and blocked in 5% normal donkey serum. Slides were then incubated overnight at room temperature with primary antibodies: CLDN1 (1:500, Bioworld) or Cx43 (1:200, Sigma-Aldrich). The following day, tissues were washed, blocked, and incubated in the dark for 1-2

hours at room temperature in secondary antibodies: donkey anti-rabbit Cy3 (1:200, Jackson ImmunoResearch) or donkey anti-rabbit 594 (1:200, Jackson ImmunoResearch). Stained tissues were imaged on a Nikon E800 fluorescence microscope with an Orca CCD camera (Hamamatsu) and Metamorph software (Molecular Devices).

Cell culture

Ishikawa cells were incubated at 37°C containing 5% CO_2 and cultured in phenol red-free DMEM/F12 (Gibco) + 10% fetal bovine serum (FBS) + 1x penicillin/streptomycin. Ishikawa cells transepithelial resistance was determined used an epithelial volt/ohm meter (World Precision Instruments). The collection of primary human endometrial stromal cells (hESCs) from consenting healthy women was approved by the University of North Carolina at Chapel Hill Institutional Review Board. Human ESCs were incubated at 37°C containing 5% CO_2 and cultured in DMEM/F12 (Gibco) + 10% FBS + 1x penicillin/streptomycin. Human ESCs were artificially decidualized for 5 days in DMEM/F12 + 2% FBS + 1x penicillin/streptomycin + 0.5 mM 8-Br-cAMP (Sigma-Aldrich) + 1 μM medroxyprogesterone 17-acetate (Sigma-Aldrich).

Water permeability assay

Ishikawa cells were plated on collagen-coated 12 mm transwells with 0.4 μm pores (Corning) and grown to confluency. Water permeability was measured as previously described.[24] In brief, Ishikawa cells were incubated with 3 μM calcein-AM (ThermoFisher) for 30 minutes prior to pre-treatment with a vehicle control or 10 nM AM (American Peptide Company/Bachem) for one hour. Ishikawa cultures were then placed in an Attofluor® Cell Chamber with 100 μL of modified Ringer solution added serosally [101 mM NaCl, 12 mM $NaHCO_3$, 24 mM HEPES, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2 \cdot 2H_2O$, 5.2 mM KCl, 10 mM D-(+)-Glucose (Sigma)]. 100 μL of isotonic Ringer solution was added apically and a baseline image obtained. After apical addition of 100 μL of Ringer solution containing an additional 150 mM NaCl (600 mOsm total osmolarity), cultures were imaged on a Leica SP5 confocal

microscope over a 30 second period using a 40x water immersion lens (0.9 NA) with calcein excited at 488 nm. Cell height and fluorescence were measured in ImageJ (NIH), and linear regression was performed on the percent change in cell height and fluorescence over time after addition of hypertonic buffer.

Ussing chamber studies

Confluent Ishikawa cultures in collagen-coated 12 mm Snapwell inserts with 0.4 μm pores (Corning) were pre-treated with a vehicle control or 10 nM AM (American Peptide Company/Bachem) before being mounted in Ussing chambers (Physiologic Instruments). Data was collected with Acquire and Analyze software (Physiologic Instruments). Cultures were bathed in 5 mL of isotonic 37°C Krebs-Ringer bicarbonate buffer circulated with 95% O_2-5% CO_2 gas on both apical and basolateral sides. Cultures were voltage-clamped and short circuit current (I_{SC}) and transepithelial resistance (TER) were measured at baseline and after addition of 100 μM amiloride apically, 10 μM forskolin apically and basolaterally, 10 μM CFTR inhibitor-172 apically, and 10 μM UTP apically (Sigma-Aldrich).

Scrape loading assay

Human ESCs were plated in a 6-well plate, grown to confluency, and artificially decidualized as described above. Human ESCs were treated with water, 10 nM AM (American Peptide Company/Bachem), 100 μM carbenoxolone (Sigma-Aldrich), or 10 nM AM + 100 μM carbenoxolone for 30 minutes. Lucifer yellow (Sigma-Aldrich) was added to each well to a final concentration of 0.05%. Decidualized hESC monolayers were then scraped with a 27 gauge needle, incubated at 37°C for 30 minutes, and then fixed in 4% paraformaldehyde for 20 minutes. Cells were then washed and stored in PBS for imaging on an Olympus IX81 (Olympus). Images were analyzed in ImageJ (NIH) by measuring the distance from the scratch to the farthest lucifer yellow dye-positive nucleus in the field. Images were converted to black and white and then inverted in ImageJ for publication. All alterations to brightness and contrast were applied to the entire image.

Statistics

All data are presented as mean ± SEM unless otherwise noted. All statistics were performed in Prism 5 (GraphPad Software, Inc.). Implantation sites per horn following blastocyst transfer were compared by Mann-Whitney test, as the AM-treated group fails the Shapiro-Wilk test of normality. Embryo spacing was analyzed by unpaired t-test with Welch's correction, as the vehicle-treated group has a very wide dispersion compared to the other two groups. Pinopode number, size, and density were significantly different by Kruskal-Wallis test, but unpaired t-tests are displayed to emphasize abrogation of the AM effect by co-treatment with its inhibitor, AM(24-50). The pinopode time course was analyzed by two-way ANOVA followed by Bonferroni post-tests. Uterine wet:dry weight; Ishikawa cell short-circuit current; distance traveled by lucifer yellow dye in the scrape loading assay; and percent penetration to the embryo in the PDZ barrier permeability assay were compared by unpaired t-tests, as we did not necessarily expect large effect sizes. Ishikawa cell transepithelial resistance (TER) over time in culture was analyzed by two-way ANOVA followed by Bonferroni post-tests. If $p<0.05$, data sets were considered statistically different.

Results

AM treatment improves implantation success and spacing

Given the subfertility of *Adm*$^{+/-}$ female mice, we first tested whether treating the uterus with AM could support implantation and early pregnancy using an in vitro fertilization (IVF) embryo transfer model. We treated the uterus of wild type female mice with AM immediately prior to blastocyst transfer and observed a significant increase in the implantation numbers between vehicle- and AM-treated animals at e6.5, an effect that was reversed by co-treatment with the mouse AM inhibitor AM(24-50) (Figure 2-1A and B). We then attempted to bolster the effect of AM by co-treating with its binding partner, complement factor H (CFH), as has been done by other groups in the context of sepsis [25,26].

However, we found that co-treatment of AM and CFH was antagonistic to implantation and led to fewer viable implantation sites at e6.5 compared to vehicle-treated animals (data not shown).

Our previously published embryonic spacing defect in the *Adm*$^{+/-}$ sub-fertile female mice then prompted us to compare the spacing patterns of embryos of vehicle- and AM-treated animals.[12] To quantify embryonic spacing, we calculated the standard deviation of distances between the implant sites within each uterine horn (Figure 2-1C). Using this method, we found that the standard deviation was significantly lower in AM-treated uterine horns, indicating that these embryos were more evenly spaced relative to each other compared to their vehicle-treated counterparts (Figure 2-1D).

AM promotes epithelial pinopode formation

We then analyzed the luminal epithelium of vehicle- and AM-treated uteri for the presence of pinopodes: spherical protrusions of the epithelial plasma membrane into the lumen of the uterus that are present in mice and humans during the window of implantation and associated with endometrial receptivity.[27] We previously discovered that the epithelial surfaces of *Adm*$^{+/-}$ uteri present fewer pinopodes, underscoring our rationale for investigating AM-mediated effects on pinopode dynamics.[12] Scanning electron microscopy (SEM) enabled visualization of pinopodes as large as 10 μm in diameter even at low magnification (Figure 2-2A). At higher power, many smaller pinopodes of approximately 1 μm in diameter were visible in areas surrounding larger pinopodes (Figure 2-2B). Interestingly, we observed three-dimensional structures that were similarly sized as pinopodes but appeared collapsed or degenerate, raising the compelling possibility that these structures are pinopodes captured in various stages of formation or regression (Figure 2-2B, arrow). On the horizon of a plane orthogonal to the uterine luminal epithelial landscape, we captured the topography of pinopodes protruding prominently above the epithelial surface (Figure 2-2C). Upon closer inspection of a single, large pinopode, we again noted several smaller pinopodes in its

shadow as well as larger degenerate pinopodes in the surrounding area (Figure 2-2D, arrows). We were also able to visualize other ultrastructural features of the epithelium, such as cell-cell borders and small, rod-like microvilli (Figure 2-2D, arrowheads and asterisks).

We then analyzed the direct effect of AM on pinopode formation by performing a time course study, treating wild type pseudopregnant uteri for 30 minutes, 2 hours, 6 hours, or 24 hours. A 30 minute treatment of the uterus with AM significantly enhanced pinopode number as well as pinopode size (Figure 2-3A-C). Ultimately, a significantly larger percentage of the epithelial surface was covered by pinopodes in AM-treated uteri compared to vehicle-treated uteri (Figure 2-3D). We observed a complete reversal of these AM-mediated changes by co-treating with AM and its inhibitor, AM(24-50) (Figure 2-3A-D).

Quantitation of pinopode density beyond the 30 minute time point revealed a common pinopode formation and regression pattern between days 2.5-3.5 of pseudopregnancy: first, an enhancement of pinopode number between 2-6 hours, followed by a decline between 6-24 hours, and eventually returning to levels comparable to those at 30 minutes (Figure 2-4A). However, in the AM-treated uteri, we observed an early leftward shift of this curve, demonstrating the ability of AM to acutely stimulate pinopode dynamics during early pregnancy (Figure 2-4B). There was a statistically significant interaction between the effects of time and treatment on pinopode number by two-way ANOVA ($p=0.003$), and Bonferroni post-tests identified significant differences in pinopode number after 30 minutes and 6 hours of treatment.

AM accelerates uterine water transport across the epithelium

While little is understood about the dynamics of pinopode formation and function, several groups have proposed that pinopodes pinocytose uterine fluid.[28,29] Therefore, to determine whether AM-mediated pinopode formation was associated with enhanced uterine water transport in vivo, we treated wild type pseudopregnant uteri with AM and measured uterine wet:dry weight. We compared vehicle- and AM-treated uteri and, consistent with our

hypothesis, found that the wet:dry weight ratio was significantly higher in AM-treated uteri (Figure 2-5A).

We then asked whether AM enhanced the uterine wet:dry weight ratio by altering epithelial ion transport or epithelial water permeability and subsequent water transport using Ishikawa cells, which are derived from an endometrial adenocarcinoma and display a hybrid phenotype between uterine glandular and luminal epithelium.[30,31] Ishikawa cells form confluent monolayers and express an array of epithelial-specific proteins, including keratins,[32] integrins,[33] and tight junctions such as claudin 1 (*Cldn1*, CLDN1) (data not shown). Furthermore, Ishikawa cells generate an average transepithelial resistance (TER) of approximately 350 Ω after four days in culture, implying a phenotype between leaky and tight epithelia and capacity for both paracellular and transcelluar transport (Figure 2-5B).[34]

We first performed Ussing chamber studies in Ishikawa cells to assess effects on ion transport by measuring changes in epithelial short-circuit current (I_{SC}) and transepithelial resistance in response to AM and the following channel modulators: amiloride, an epithelial sodium channel (ENaC) inhibitor; forskolin, which induces cAMP activation of the cystic fibrosis transmembrane conductance regulator (CFTR); CFTR inhibitor-172; and UTP, an activator of calcium-activated chloride channels. Pre-treatment of Ishikawa cells with AM did not affect changes in I_{SC} and TER in response to this series of ion channel activators and inhibitors, indicating that AM does not affect uterine water balance by altering ion transport (Figure 2-5C and D). Notably, modulation of CFTR activity conferred negligible changes in I_{SC} and TER, which is consistent with low expression of *CFTR* in Ishikawa cells (data not shown).

We then assessed whether AM affects water permeability of the Ishikawa cell apical membrane by confocal microscopy. Following addition of a hypertonic buffer solution on the apical Ishikawa membrane to create an osmotic gradient, we observed a significant enhancement in the rate of both Ishikawa cell shrinkage and calcein fluorescent

intensification with AM pre-treatment compared to vehicle pre-treatment, indicating that AM enhances the rate of water transport across the apical surface of Ishikawa cells (Figure 2-5F and G). Altogether, these data support a role for AM in primary water transport, but not ion transport, in the uterus.

AM ensures appropriate epithelial CLDN1 localization

The junctional protein CLDN1 plays essential roles in water homeostasis in epithelial barriers. For example, genetic deletion of *Cldn1* in mice confers lethality by post-natal day 1 due to defective epidermal barrier formation and consequent water loss.[35] Previously published associations between epithelial cell junction integrity and fertility prompted us to examine the localization of CLDN1.[16,18] Therefore, we employed our murine model of *Adm* haploinsufficiency and subfertility to investigate the effect of AM on CLDN1 localization in the pregnant uterine epithelium. In wild type pregnant uteri, we observed continuous CLDN1 expression from the apical to the basolateral sides of the lateral membranes of epithelial cells. However, in $Adm^{+/-}$ pregnant uteri, we noted collapse of CLDN1 to the basolateral side of the lateral epithelial membranes (Figure 2-6). In all animals of both genotypes, we observed circumferentially patchy expression of CLDN1 around the lumen. These data indicate that even a modest change in the local production of AM in the uterus can influence the localization of epithelial barrier proteins during the peri-implantation period.

AM enhances stromal gap junction communication and barrier integrity

We next turned our attention to uterine stroma, which includes the primary decidual zone (PDZ) adjacent to the embryo as an additional site of robust *Adm* and cell junction expression during the peri-implantation period.[11] Decidualized stromal cells communicate with each other to organize this complex, three-dimensional structure, which provides an early protective barrier to promote successful implantation. Accordingly, we confirmed that the gap junction protein Cx43, which facilitates inter-cellular communication, is highly expressed in the early implantation site using three-dimensional optical projection

tomography (Figure 2-7A and B). To assess the direct effect of AM on Cx43 in uterine stroma, we treated pseudopregnant uteri with AM and performed immunohistochemistry for Cx43, finding that AM treatment enhanced Cx43 expression in stroma adjacent to the luminal epithelium – the future site of the PDZ (Figure 2-7C).

We then functionally evaluated whether AM could influence gap junction coupling between stromal cells by using primary human endometrial stromal cells (hESCs) to perform a scrape loading assay. In this assay, cells are “scratched” open, and the distance of transfer of a gap junction-permeable dye, lucifer yellow, is measured across the monolayer. Interestingly, AM promoted functional gap junction coupling between hESCs as evidenced by the enhanced distance of lucifer yellow dye transfer across the cell monolayer. This effect was abrogated by co-treatment with the gap junction inhibitor carbenoxolone (Figure 2-7D and E).

We hypothesize that communication between stromal cells via gap junctions provides cues that direct the formation of the PDZ, which forms a selective barrier around the embryo. Therefore, given the effect of AM on cellular communication, we assessed the consequences of a reduction in AM peptide on the selectivity of this barrier in vivo. We examined the integrity of the PDZ in wild type and *Adm*$^{+/-}$ female mice by injecting pregnant mice retro-orbitally with biotinylated bovine serum albumin (BSA) and then assessing the depth of BSA penetration into the decidua toward the embryo on two different axes of the implantation site. In *Adm*$^{+/-}$ females, BSA penetrated significantly closer to the embryo compared to wild type animals, indicating compromised PDZ barrier function in heterozygous decidua (Figure 2-7F and G). Altogether, these data serve as evidence for a role for AM in stromal cell communication and PDZ barrier formation in support of early pregnancy and point to additional mechanistic insight into how AM promotes pregnancy success.

Discussion

Here, we have elucidated molecular and cellular effects of AM on the epithelial and stromal compartments of the uterus during the peri-implantation period. Moreover, we demonstrate that administration of AM directly into the uterus enhances the embryo implantation rate in mice after blastocyst transfer, suggesting a potential use for AM peptide in the context of embryo transfer or IVF in humans.

There is intense interest in improving the IVF live birth rate, which currently stands at less than 50%. Advances in clinical embryology have enabled the identification of aneuploid embryos prior to transfer, but the IVF live birth rate remains low because we do not completely understand uterine factors supporting implantation. Despite this lack of understanding, attempts have been made to overcome endometrial receptivity defects. For example, systemic G-CSF treatment has been shown in a single randomized controlled trial to benefit women with recurrent implantation failure but not provide benefit to other women.[36] Endometrial scratching, in which the endometrium is physically injured to provoke an inflammatory response, is currently being recommended by practitioners in some countries to many women refractory to IVF.[37] However, the mechanisms of action following this invasive procedure remain unclear. Furthermore, inflammation, if uncontrolled, may be associated with abnormal implantation and pregnancy complications.[38] Therefore, direct, short-term treatment of the endometrium with AM peptide may provide an attractive and more efficacious alternative to either a systemically administered drug or an inflammation-provoking injury.

Unexpectedly, we found that co-administration of AM and CFH, which binds and stabilizes AM in plasma, was antagonistic to embryo implantation.[39] In addition to associating with AM, CFH blocks activation the alternative complement pathway, deterring aberrant complement deposition by facilitating the distinction between self and non-self cells.[40] Unfortunately, very little is known about the role of complement in embryo

implantation and the establishment of pregnancy. Recently, two groups found that activation of complement is associated with embryo loss in both mice and humans.[41,42] As we begin to appreciate the pro-inflammatory environment of embryo implantation, we believe it is possible that exogenous administration of high levels of CFH caused dysregulation of fine-tuned, physiological complement and inflammatory responses that are critical for implantation success.[43]

Notably, in our study, AM improved not only the implantation rate but also the spacing between embryos *in utero*. How embryos space within the mouse uterine horn is not completely understood and, at first glance, appears irrelevant for singleton human pregnancies.[44] However, what we learn about embryo spacing can certainly inform our understanding of embryo-uterine interactions and the choice of implantation location, which are highly relevant for both singleton and multi-fetal gestations, especially given the programming of clinical complications of pregnancy by implantation defects.[2]

Subsequently, we found that AM treatment of the uterus promotes pinopode formation, size, and epithelial surface coverage. Only a handful of descriptive studies provide information on pinopodes, with the average mouse pinopode quantified at approximately 6 μm in diameter.[45] Indeed, we identified pinopodes at least 6 μm in diameter, but the majority of pinopodes we observed were approximately 1 μm in diameter. These differences in size could be explained by differences in genetic backgrounds, by visualization of the uterus at different time points during pregnancy, or by different effects of pseudopregnancy. We frequently observed what appeared to be ruptured pinopodes in various stages of growth or regression. Ruptured pinopodes have been observed in both rodents and humans, but more frequently in the latter species.[46,47] Other groups have argued that pinopode coverage of the epithelium ranges from 0.6% to 20%;[45] our studies were consistent with these findings.

Pinopodes may pinocytose uterine fluid, ultimately affecting uterine water balance in support of implantation, though this hypothesis has been largely supported in rats and less so in humans.[28,29,48,49] We found that AM accelerated water loss across the apical Ishikawa cell membrane in the context of hypertonic stress, suggesting that AM renders the apical Ishikawa cell membrane more permeable to water. Early in the peri-implantation period, uterine water imbibition causes an estrogen-induced accumulation of water in the uterine lumen via the transcellular pathway, ultimately changing luminal fluid viscosity to facilitate an environment amenable to implantation; this occurs in part by altering aquaporin expression and availability[50-52] In the mouse, expression of aquaporins-2, -3, and -4 has been observed in uterine luminal epithelium, suggesting that these aquaporins may participate in the accumulation of water in the uterine lumen.[51,52] However, at the time of implantation, water transport also occurs in the opposite direction at the time of closure of the uterine epithelium around an implanting blastocyst.[53] Therefore, water transport across the uterine luminal epithelium occurs bidirectionally during the peri-implantation period.

Our findings of enhanced uterine wet:dry weight and accelerated water transport across Ishikawa cells with AM treatment show that AM may contribute to this water transport to promote implantation. In further support of this idea, estrogen is a potent regulator of uterine water transport, aquaporins, and *Adm* gene expression – both directly through estrogen response elements in the 5' untranslated region and indirectly through an estrogen-induced cohort of miRNAs targeting the 3' untranslated region of the Adm gene.[54,55]. Given the uterine wet:dry weight data, it is likely that AM stimulates water transport from the uterine lumen to the uterine wall, possibly through pinopodes, although the tissue compartments in which this water accumulates have yet to be determined. The uterine wet:dry weight may also be explained in part by effects of AM on circulatory and lymphatic vascular systems, but the direct vascular effects on the uterus in our AM-supplemented blastocyst transfer model are currently unknown.

Given the lethal water loss phenotype of mouse embryos lacking the tight junction protein CLDN1, we utilized our sub-fertile *Adm*$^{+/-}$ mice to investigate the effect of AM on CLDN1 localization in the uterine epithelium. Analysis of sub-fertile *Adm*$^{+/-}$ uterine epithelium suggested a role for AM in appropriate localization of CLDN1. Notably, this is not the first association between aberrant cell junctional protein expression and localization and endometrial receptivity defects. An epithelial-specific deletion of *Stat3* and a pan-uterine deletion of *Msx1/Msx2* on day 4 of pregnancy or pseudopregnancy, respectively, both demonstrate anomalous CLDN1 expression and infertility.[16,18] More specifically, in each of these transcription factor loss-of-function studies, CLDN1 was persistently expressed in epithelium, while it was absent in floxed control animals. Therefore, Pawar and colleagues concluded that the repression of *Cldn1* facilitates a requisite loss of cell polarity during early implantation.[16] Our observations of aberrant CLDN1 localization in epithelium heterozygous for *Adm* support a role for AM – likely through second messenger signaling – in epithelial cell organization, which has consequences for endometrial receptivity and epithelial remodeling during implantation.

In the present study, we also investigated effects of AM on uterine stroma during peri-implantation, concluding that AM promotes Cx43 expression in the uterus, stromal intercellular communication, and organization of the PDZ. Our conclusion concerning the effect of AM on Cx43 expression is consistent with our previously published study in lymphatic endothelium.[22] The importance of Cx43 and gap junction-mediated intercellular communication in the uterus is highlighted by the effects of conditional knockout of Cx43 in the uterus, which negatively affects decidualization, expression of angiogenic factors, and placental angiogenesis, ultimately leading to fetal loss.[56] Furthermore, *Adm* gene expression is upregulated two-fold in stroma from *Msx1*$^{d/d}$*/Msx2*$^{d/d}$ uteri, possibly in a compensatory fashion to support the implantation defects in these animals.[18]

Together, the effects of AM on pinopodes, water permeability, and cell junctions in the epithelial and stromal compartments of the uterus during peri-implantation synergize to support implantation, as evidenced by our finding that treatment of the mouse uterus with AM prior to blast transfer improves the implantation rate. This discovery complements elegant studies performed by Yallampalli and colleagues in rats, finding that continuous administration of the human AM inhibitor, AM(22-52), via osmotic mini-pumps is detrimental to implantation and placentation.[57,58] Similarly, genetic loss of *Adm* expression in the fetal placenta causes under-recruitment of uterine natural killer (uNK) cells and a loss of spiral artery remodeling, phenotypes consistent with the development of preeclampsia, a dangerous placenta-driven complication of pregnancy.[59] Consistent with murine data, we previously found the biomarker mid-regional pro-adrenomedullin (MR-proADM) to be reduced in women with severe preeclampsia and a polymorphism within the human *Adm* gene to be associated with reduced birth weight, highlighting its relevance to human pregnancy.[60,61] In summary, our findings provide molecular insights into the compartmental effects of AM signaling in the uterus during peri-implantation and highlight a potential therapeutic for poor implantation, possibly in the context of ART. Most importantly, studies addressing the safety and efficacy of intra-uterine delivery of AM in humans are merited and may have the potential to improve fertility rates.

FIGURES

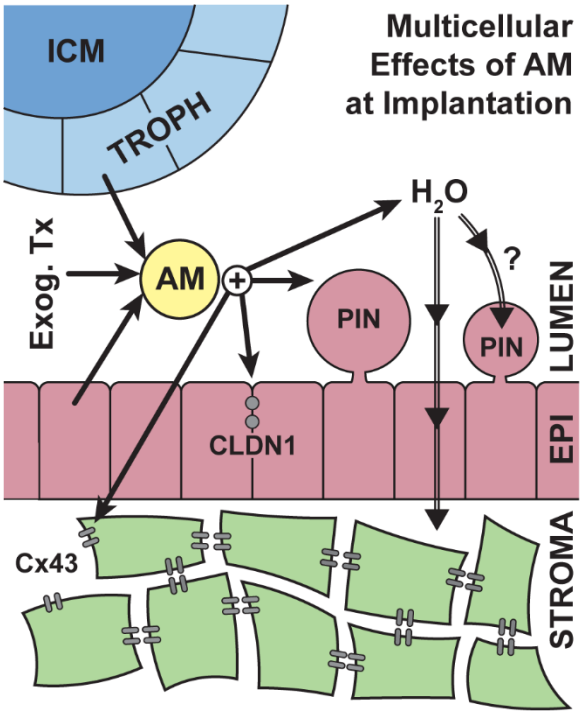

Figure 2-A1. Graphical abstract. AM, adrenomedullin; CLDN1, claudin 1; Cx43, connexin 43; EPI, epithelium; ICM, inner cell mass; PIN, pinopode; TROPH, trophoblast.

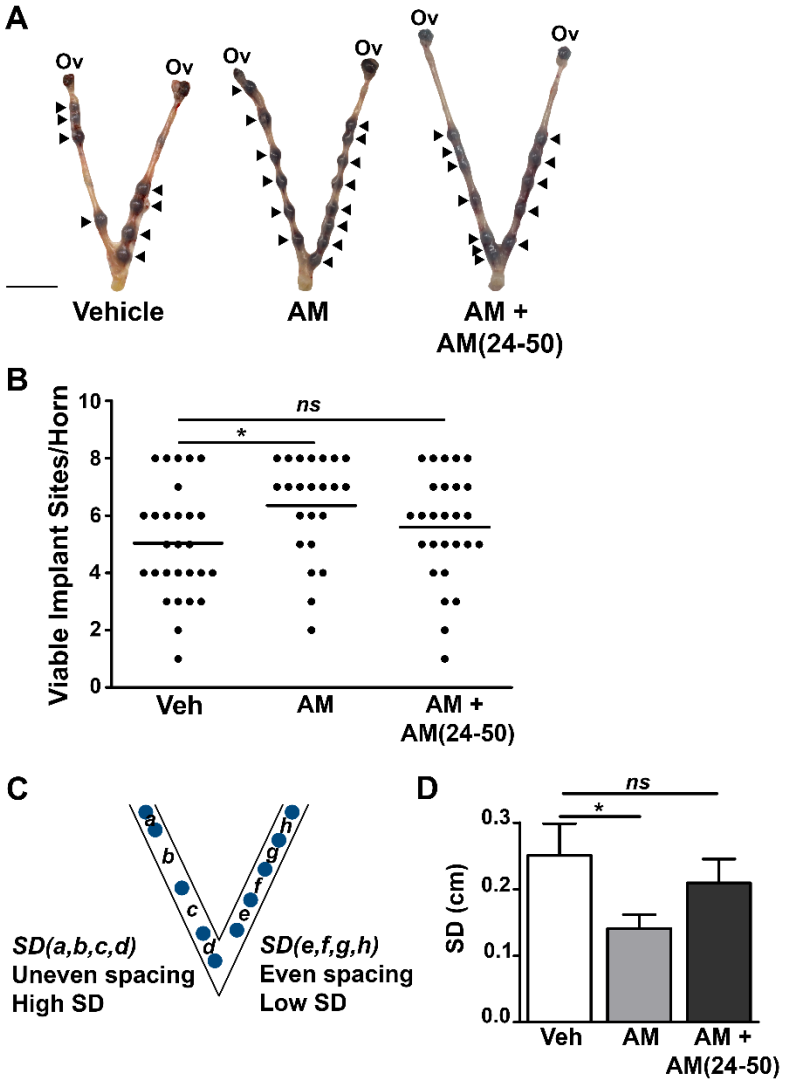

Figure 2-1. AM improves implantation success and spacing in mice. A, Images of e6.5 embryos within uteri treated with vehicle, AM, or AM + AM(24-50) prior to transfer of 8 blastocysts per horn. Arrowheads indicate viable implantation sites as determined by uptake of Evans blue dye. Scale bar, 1 cm. B, Quantitation of viable implant sites per horn. Dots represent uterine horns. *ns*, not significant. *p<0.05, Mann-Whitney test. C, Illustration of quantitation method for spacing of e6.5 embryos within uterine horns. SD, standard deviation. D, Quantitation of spacing in vehicle- (n=26), AM- (n=21), and AM + AM(24-50) (n=25) horns using method depicted in (C). *p<0.05, unpaired *t* test with Welch's correction.

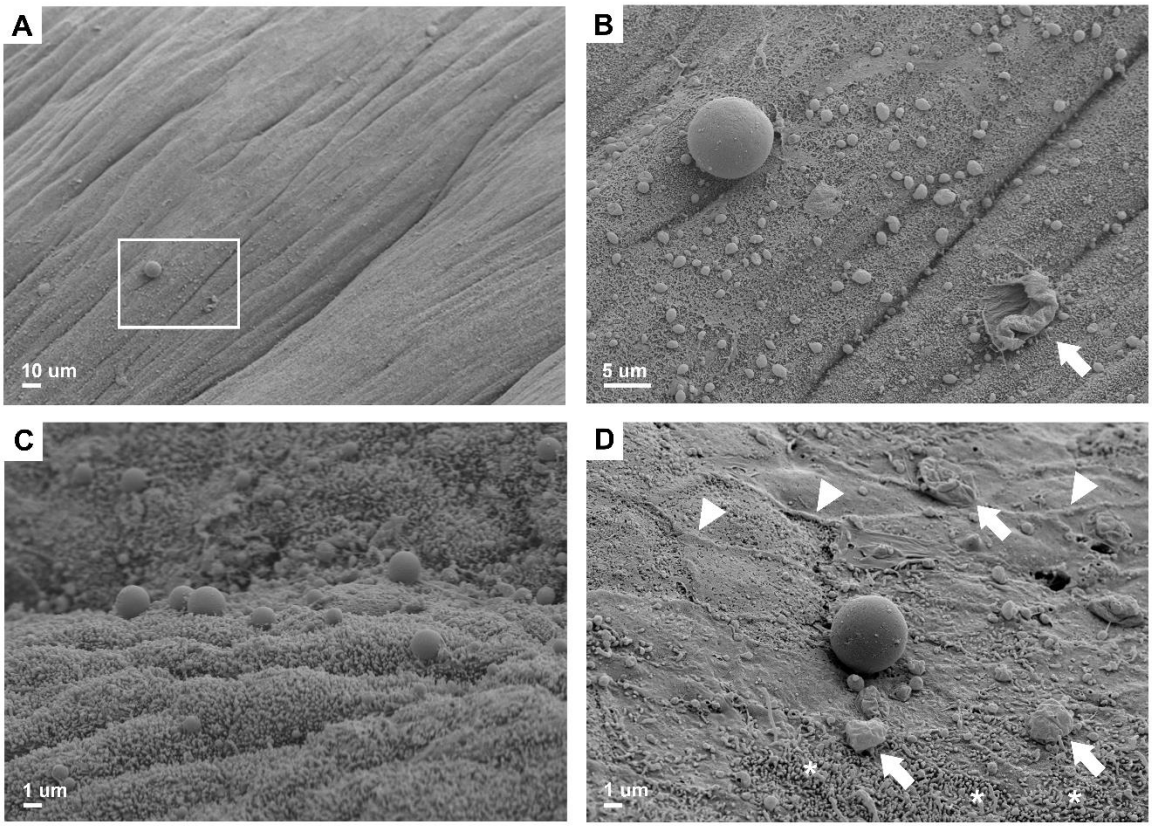

Figure 2-2. Scanning electron micrographs of pinopodes and other ultrastructure features of uterine epithelial cells in wild type mice on day 2.5 of pseudopregnancy. A, Box encloses the image displayed in (B). Scale bar, 10 μm. B, Juxtaposition of a single large pinopode and many smaller pinopodes. Arrow points to a potentially degenerate pinopode. Scale bar, 5 μm. C, Pinopodes projecting above the epithelial cell layer into the lumen of the uterus. Scale bar, 1 μm. D, Arrows point to potentially degenerate pinopodes. Arrowheads denote examples of cell-cell borders. Asterisks signify area dense in microvilli. Scale bar, 1 μm.

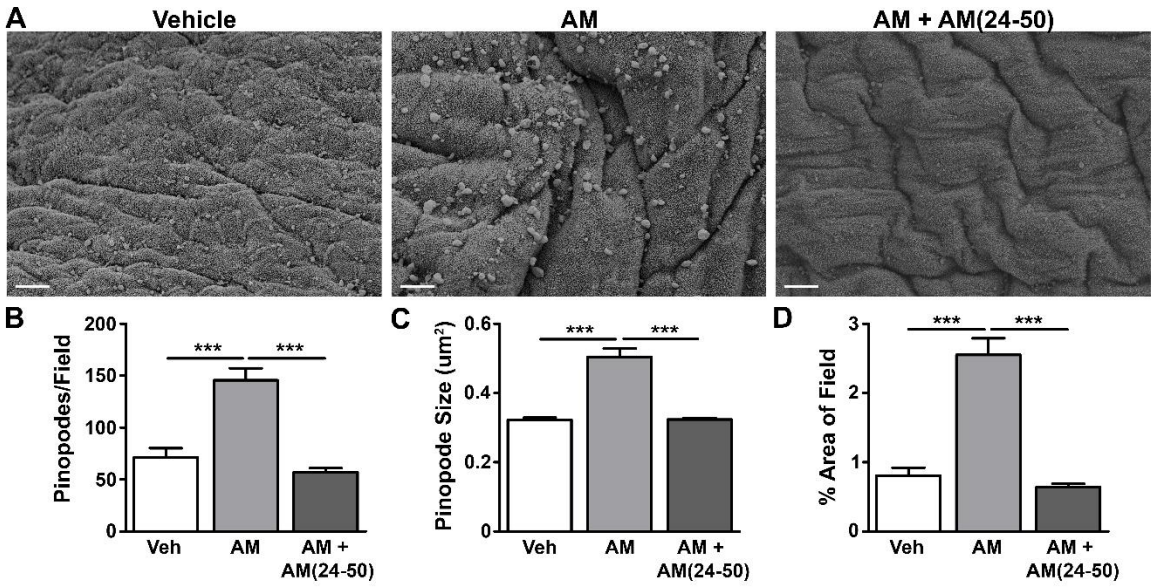

Figure 2-3. AM promotes pinopode formation and size. A, Scanning electron micrographs of pinopodes in wild type uteri treated with vehicle, AM, or AM + AM(24-50) for 30 minutes. Scale bars, 5 µm. B-D, Quantitation of pinopodes per field (B), size (C), and percent area of field (D). n≥4 fields from n≥3 animals per treatment group. ***p<0.001, unpaired *t* test.

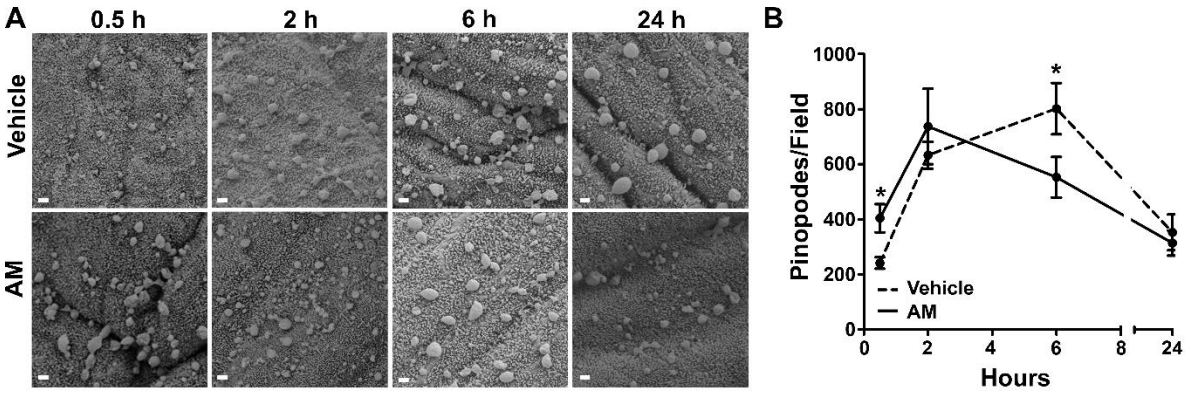

Figure 2-4. AM accelerates pinopode formation dynamics in wild type mice between days 2.5-3.5 of pseudopregnancy. A, Scanning electron micrographs of vehicle- and AM-treated uteri throughout treatment time course. Scale bars, 1 μm. B, Quantitation of pinopodes per field throughout treatment time course. n≥6 fields total from n=3 animals per treatment group. *p<0.05 at indicated time point, Bonferroni post-tests following two-way ANOVA, which describes a significant interaction between time and treatment.

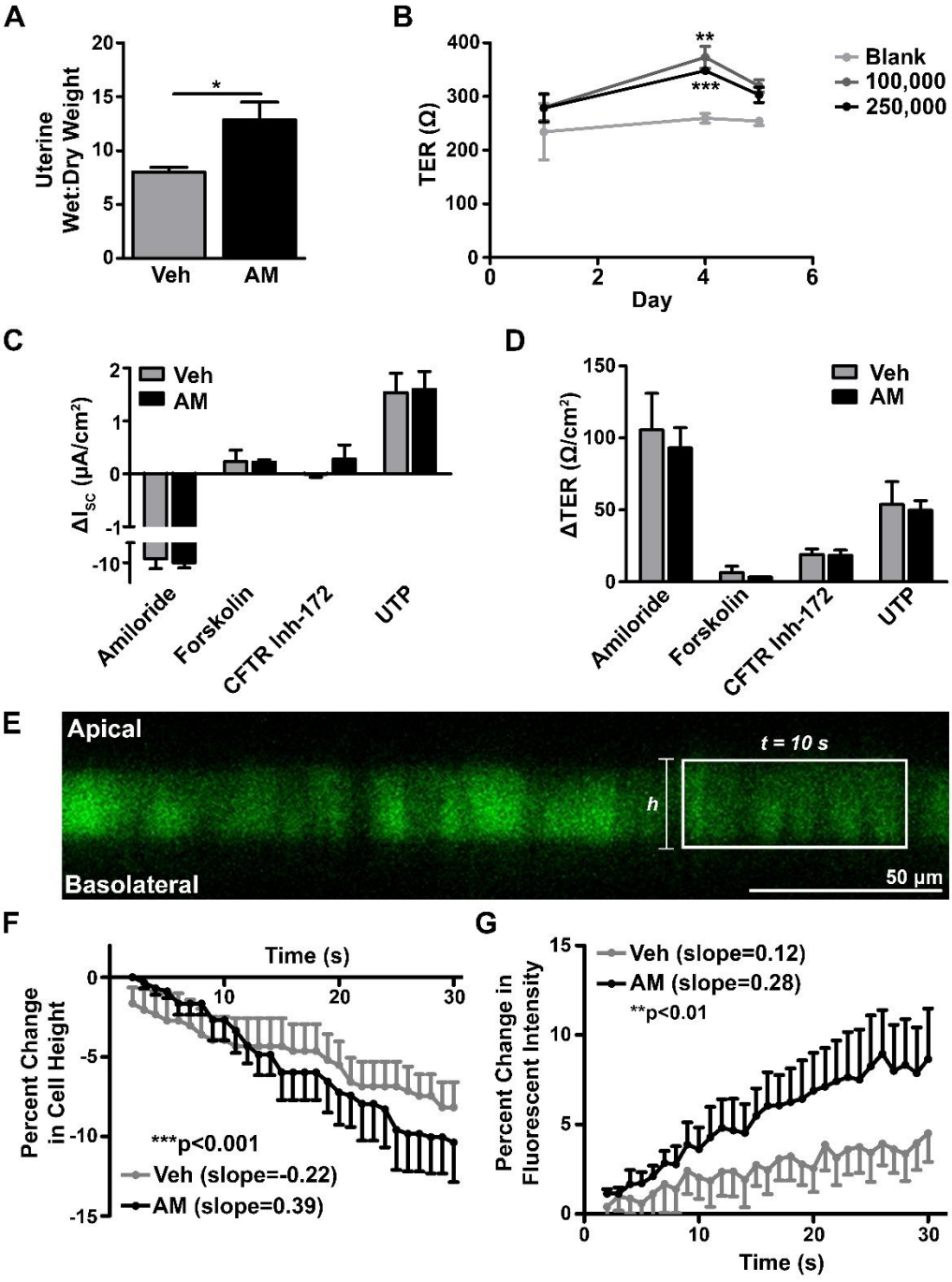

Figure 2-5. AM enhances uterine wet:dry weight in vivo and water transport across Ishikawa cells in vitro. A, Wet:dry weight ratio of wild type uteri on day 2.5 of pseudopregnancy. n=3 animals per treatment group. *p<0.05, unpaired *t* test. B, Change in transepithelial resistance (TER) of Ishikawa cells seeded at densities of 100,000 and 250,000 cells per transwell compared to a blank transwell between days 1 and 6 of culture. n≥3 cultures per time point. **p<0.01, ***p<0.001, Bonferroni post-tests at each density compared to blank after two-way ANOVA. C and D, Changes in short-circuit current (C) and TER (D) after addition of activators and inhibitors of ion channels following vehicle- and AM-pre-treated

cultures (n=3 per pre-treatment group). E, Representative image of calcein-loaded Ishikawa cells used in water permeability experiment. Rectangle encloses representative series of adjacent cells analyzed for changes in cell height and fluorescence after addition of hypertonic solution. Scale bar, 50 μm. F, Percent change in Ishikawa cell height after vehicle (n=5 cumulative treatments from 3 cultures) and AM (n=6 cumulative treatments from 3 cultures) pre-treatment followed by hypertonic shock. Data is presented as mean + SEM (vehicle) or mean – SEM (AM). Slopes were calculated by linear regression analysis. ***p<0.001, ANCOVA. G, Percent change in calcein fluorescent intensity after hypertonic shock. Data is presented as mean – SEM (vehicle) or mean + SEM (AM). Slopes were calculated by linear regression analysis. **p<0.01, ANCOVA.

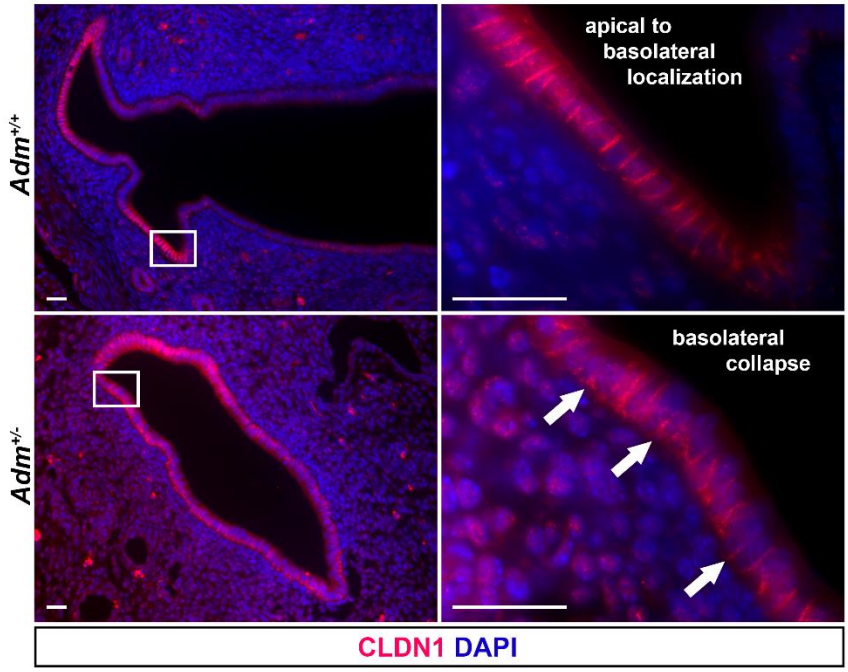

Figure 2-6. AM contributes to proper CLDN1 localization. Immunohistochemistry for CLDN1 in wild type (n=3) and *Adm*$^{+/-}$ (n=4) e5.5 inter-implantation sites. Arrows denote CLDN1 localization, spanning the apical-basolateral axis of epithelial cell lateral borders (*Adm*$^{+/+}$) or concentrated on the basolateral side of the epithelial cell layer (*Adm*$^{+/-}$). Scale bars, 20 μm.

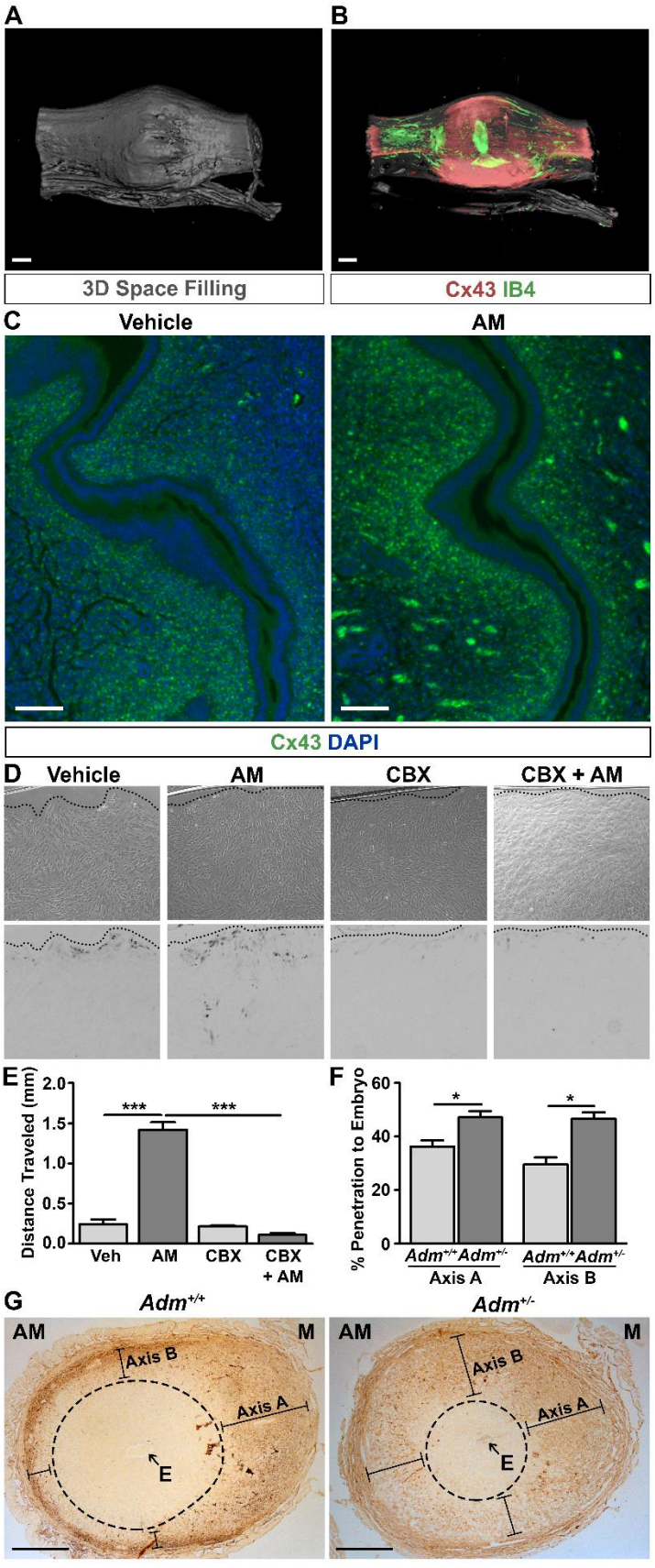
A
B
3D Space Filling
Cx43 IB4
C
Vehicle
AM
Cx43 DAPI
D
Vehicle
AM
CBX
CBX + AM
E
Distance Traveled (mm)
2.0
1.5
1.0
0.5
0.0
***
***
Veh
AM
CBX
CBX + AM
F
% Penetration to Embryo
60
40
20
0
*
*
Adm+/+ Adm+/-
Axis A
Adm+/+ Adm+/-
Axis B
G
Adm+/+
Adm+/-
AM
M
Axis B
Axis A
E
AM
M
Axis B
Axis A
E

Figure 2-7. AM enhances gap junction expression and communication in uterine stroma. A and B, Optical projection tomography (OPT) three-dimensional space filling scan (A) and Cx43 expression scan (B) of a wild type e5.0 implantation site and adjacent inter-implantation sites. IB4 lectin staining indicates endothelium and embryonic tissue. Scale bars, 400 μm. C, Immunohistochemistry for Cx43 in uteri treated with vehicle or AM for 30 minutes. Clusters of signal at the periphery of the AM image are autoflurescent red blood cells. n=3 animals per treatment group. Scale bars, 100 μm. D, Bright field (top) and inverted fluorescent (bottom) images of a scrape loading assay in hESCs. Dotted lines indicate location of the scrape. E, Quantitation of distance traveled by lucifer yellow dye from the scrape. CBX, carbenoxolone, a gap junction inhibitor. n=3 fields per treatment group. ***p<0.001, unpaired *t* test. F, Quantitation of distance traveled by biotinylated BSA toward the embryo of e7.5 $Adm^{+/+}$ (n≥7 per axis) and $Adm^{+/-}$ (n≥25 per axis) implantation sites as a percentage of the total length of the axes depicted in (A). *p<0.05, unpaired *t* test. G, Representative images of e7.5 $Adm^{+/+}$ and $Adm^{+/-}$ implantation sites stained for diaminobenzidine to assess the penetration of biotinylated BSA toward the embryo. The area within the dashed ellipses represents the zone not penetrated by BSA. Lines represent distance traveled by BSA toward embryo and quantitated in F. Scale bars, 0.5 mm. AM, anti-mesometrial side; E, embryo; M, mesometrial side.

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Chapter 3: MR-proADM is a Potential Biomarker of Endometriosis, an Infertility-Associated Disease[1]

Overview

Objective: To test adrenomedullin (*Adm*, AM) as a downstream target of STAT3 in endometrial cells and to test mid-regional pro-adrenomedullin (MR-proADM) as a biomarker of endometriosis.

Design: Cross-sectional analysis of adrenomedullin (*Adm*, AM) expression in eutopic endometrium and of MR-proADM in plasma from women with and without endometriosis. Prospective study of MR-proADM levels in women with endometriosis undergoing surgical resection of ectopic lesions.

Setting: Academic medical centers in the United States.

Patients: 15 patients with endometriosis and 11 healthy controls who donated eutopic endometrial biopsies; 28 patients with endometriosis and 19 healthy controls who donated plasma for MR-proADM analysis.

Intervention(s): None.

Main Outcome Measure(s): Adm mRNA levels by qRT-PCR after activation of STAT3 by IL-6 in Ishikawa cells. Immunohistochemistry for AM in eutopic endometrial biopsies from women with endometriosis compared to healthy donors. MR-proADM levels measured by

[1]Under Review: Matson BC, Quinn KE, Lessey BA, Young SL, & Caron KM. Mid-regional pro-adrenomedullin is a potential biomarker of endometriosis.

commercial immunoassay in plasma from healthy women and women with endometriosis who subsequently underwent surgical resection of ectopic lesions.
Result(s): Activation of STAT3 by IL-6 upregulated *Adm* mRNA expression in Ishikawa cells. AM protein levels were elevated in the eutopic endometrium of women with endometriosis. MR-proADM concentrations were higher in women with endometriosis but were not correlated with disease stage, corrected by surgery, or predictive of fertility outcome.

Introduction

Endometriosis is a common gynecological disease characterized by the presence of endometrial tissue outside of the eutopic endometrium, commonly in the peritoneum of the pelvis and in pelvic organs, often causing scarring and pain.[1,2] Although endometriosis is also strongly associated with subfertility and infertility, the mechanisms underlying fertility problems in women with stage 1 to 2 disease is a subject of debate in the field.[3,4] The non-specific nature of endometriosis symptoms makes the disease difficult to diagnose, and confident diagnosis usually requires visualization of ectopic lesions at surgical exploration. Endometriosis is equally difficult to treat; hormonal therapies and surgical excision of ectopic lesions are the mainstays of treatment but are not always effective.[5,6] These diagnostic and therapeutic challenges have fueled interest in identifying biomarkers and signaling pathways associated with endometriosis.[7]

Recently, Kim et al. demonstrated aberrant activation of signal transducer and activator of transcription 3 (STAT3) in the eutopic endometrium of women with endometriosis.[8] Other studies consistently point to endometriosis-associated factors that affect STAT3 activation: the cytokine interleukin 6 (IL-6), which activates STAT3 through the IL-6 receptor, is elevated in the peritoneal fluid of women with endometriosis;[9-11] miR120, which targets STAT3, is elevated in endometriotic cyst stromal cells;[12] and protein inhibitor of activated STAT3 (PIAS3) and dual-specificity phosphatase-2 (DUSP2), negative

regulators of STAT3, are downregulated in endometriosis.[13,14] Kim et al. also found hypoxia inducible factor 1A (HIF1A), which is stabilized by STAT3, to be elevated in the eutopic endometrium of women with endometriosis.[8] Taken together, these data strongly implicate the IL-6-STAT3-HIF1A pathway in the pathophysiology of endometriosis.

Both STAT3 and HIF1A have been previously identified as regulators of adrenomedullin (*Adm*, AM), a versatile peptide hormone.[15-18] AM is expressed in the female reproductive system and has been associated with female reproductive physiology, including embryo implantation and placentation, and in pathophysiology, including subfertility and complications of pregnancy like preeclampsia.[19,20] In endometriosis, AM has been found to be higher in intrafollicular fluid and negatively associated with oocyte maturity and embryo quality in women with endometriosis, underscoring a potential link between AM, endometriosis, and fertility.[21] Collectively, these data imply that AM may be able to serve as a biomarker of endometriosis.

Mid-regional pro-adrenomedullin (MR-proADM) is a byproduct of post-translational processing of pre-pro-AM peptide and is a more stable analyte than the mature AM peptide.[22] In the past decade, many groups have found prognostic value for MR-proADM plasma concentrations as a biomarker of heart failure,[23] community-acquired pneumonia,[24] and sepsis,[25] among other diseases. In reproduction, MR-proADM has been tested as a biomarker of gestational diabetes and preeclampsia.[26,27] Here, we test the hypothesis that MR-proADM, as a surrogate for AM potentially downstream of the IL-6-STAT3 axis, can serve as a biomarker of endometriosis.

Materials and Methods

Study Design and Human Subjects

The study was approved by the Institutional Review Boards of Greenville Health System, Michigan State University, and the University of North Carolina at Chapel Hill.

Informed consent was obtained from all study participants, who were between the ages of 18 and 45 and had not used hormonal therapies or an intrauterine device in the three months preceding biopsy or plasma collection. Eutopic endometrial biopsies were collected from healthy donor women and women with endometriosis in both proliferative and secretory phases at the time of surgery at Greenville Health System and the University of North Carolina. Plasma samples for analysis of MR-proADM concentrations were collected from healthy women and from women with endometriosis in both proliferative and secretory phases at Greenville Health System and the University of North Carolina. Patients who wished to conceive were followed expectantly after surgery for up to 6 months and pregnancies recorded. Pregnancy was defined as a visible gestational sac on ultrasound with cardiac activity and referral for obstetrical care. The clinical characteristics of women from whom plasma was collected is displayed in Table 3-1. MR-proADM concentrations were measured in undiluted plasma using a commercial assay (BRAHMS MR-proADM KRYPTOR) by Phadia Immunology Reference Laboratory.

Immunohistochemistry

Five micron sections of paraffin-embedded endometrial biopsies were deparaffinized and hydrated. Following antigen retrieval in 10 mM citric acid/0.05% Tween 20, pH 6.0, endogenous peroxidase activity was quenched with 3% hydrogen peroxide in phosphate buffered saline (PBS). Tissues were permeabilized with PBS/0.1% Triton X-100 (PBST) and then blocked in 10% normal goat serum/1% bovine serum albumin in PBST. Tissues were incubated in anti-adrenomedullin primary antibody (1:200, Abcam ab69117) in block overnight at room temperature. The following day, slides were washed and incubated in biotinylated goat anti-rabbit (1:250, Jackson ImmunoResearch) for one hour. Avidin-biotin complexes (VECTASTAIN Elite ABC Kit, Vector Laboratories) were added to tissues for 30 minutes, and then diaminobenzidine (DAB Peroxidase (HRP) Substrate Kit, Vector Laboratories) was added for two minutes. Slides were rinsed with tap water, counterstained

with hematoxylin (Vector Laboratories) for 20 seconds, and then rinsed with tap water again. Tissues were dehydrated and then coverslipped using DPX mountant (VWR). Slides were imaged on a Zeiss AxioImager with ProgRes CapturePro software (Jenoptik). Staining intensity was determined by a blinded observer (KEQ) and graded on a scale of 0 (no staining) to 4 (strong staining).

Cell Culture, Western Blot, and qRT-PCR

Ishikawa cells were cultured in DMEM/F12 (Gibco) + 10% fetal bovine serum (FBS) + 1x penicillin/streptomycin (Gibco) in a 37°C incubator containing 5% CO_2. For western blot analysis, Ishikawa cells were grown to confluency in 10 cm dishes and were treated with a vehicle control or 1, 10, or 100 ng/mL human IL-6 (R&D Systems) for 15 minutes. Cells were lysed in PBS + 10 nM NaF + 2 mM $Na_3(VO_4)$ + 2mM PMSF + protease inhibitor cocktail, and protein concentration in the lysates was determined using a BCA Protein Assay Kit (Pierce). Twenty micrograms of protein per sample were loaded on a SDS-PAGE gel (Bio-Rad) and then transferred to a nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked in either 5% bovine serum albumin (Fisher Scientific) or 5% nonfat dry milk for one hour at room temperature and then incubated overnight at 4° in primary antibodies: phospho-STAT3 (1:2,000 in 5% BSA, Cell Signaling) or STAT3 (1:2,000 in 5% milk, Cell Signaling). Blots were washed three times in tris-buffered saline/0.1% Tween 20 and then incubated for two hours at room temperature in secondary antibodies: DyLight 680 goat anti-mouse (1:15,000, Thermo Scientific) and DyLight 680 goat anti-rabbit (1:15,000 Thermo Scientific). Membranes were then imaged on an Odyssey CLx (LI-COR).

For gene expression analysis, Ishikawa cells were grown to near confluency in 10 cm dishes and then serum starved overnight in serum-free media. Cells were treated with 100 ng/mL IL-6 (R&D Systems) for 1 hour. RNA was collected and isolated using TRIzol (Thermo Fisher Scientific) according to the manufacturer's protocol. Complementary DNA was synthesized from 2000 ng of DNase-treated RNA using M-MLV reverse transcriptase

(Invitrogen). Quantitative real-time PCR was performed using a human *Adm* Assay on Demand (Applied Biosystems, Hs00969450_g1) and human GAPDH primers and probe (Applied Biosystems, 4310884E) on a StepOne Plus (Applied Biosystems). qRT-PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

All statistical analyses were performed in Prism 5 (GraphPad Software, Inc.). *Adm* gene expression in vehicle- and IL-6-treated Ishikawa cells was compared by unpaired t-test given the expected small effect size. AM staining intensity in endometrial compartments in biopsies from healthy women and women with endometriosis was compared by Mann-Whitney test, as the data are not normally distributed. Composite AM staining scores and MR-proADM concentrations in plasma from healthy women and women with endometriosis were normally distributed and compared by unpaired t-test. MR-proADM concentrations by menstrual cycle phase; stage of disease; surgical status; and fertility outcome were compared by Kruskal-Wallis test. Data was considered statistically significant if $p<0.05$.

Results

Activation of STAT3 by IL-6 induces Adm expression

Given elevated levels of IL-6 and phosphorylated STAT3 (pSTAT3) in peritoneal fluid and eutopic endometrium, respectively, of women with endometriosis,[9-11] and given prior evidence for STAT3 regulation of *Adm* expression,[16,17] we tested whether IL-6 could induce *Adm* expression in an endometrial cell line, Ishikawa cells. First, we treated Ishikawa cells with increasing doses of IL-6 to confirm that IL-6 induced phosphorylation of STAT3 in Ishikawa cells. Indeed, we observed an IL-6 dose-dependent increase in pSTAT3 by western blot (Figure 3-1A). We then assessed whether IL-6-mediated phosphorylation of STAT3 induced *Adm* expression, finding that treating Ishikawa cells with 100 ng/mL IL-6 for

15 minutes upregulated *Adm* gene expression approximately 1.4-fold by qRT-PCR (Figure 3-1B).

AM staining is enhanced in eutopic endometrium of women with endometriosis

Considering the elevation of pSTAT3 in endometriotic endometrium coupled with our *in vitro* evidence for STAT3-mediated upregulation of *Adm* expression in endometrial cells, we asked whether AM is also upregulated in the endometrium of women with endometriosis. Indeed, we found that AM staining was significantly greater in luminal epithelium, glandular epithelium, and stroma of eutopic endometrium from women with endometriosis compared to healthy controls (Figure 3- 2).

MR-proADM plasma levels are elevated in women with endometriosis

We then asked whether circulating plasma levels of MR-proADM, a stable precursor to the mature AM peptide, are elevated in women with endometriosis. The clinical characteristics of healthy controls and women with endometriosis who donated plasma for MR-proADM analysis are displayed in Table 3-1. First, we confirmed that MR-proADM levels are stable across all phases of the menstrual cycle in healthy women (Figure 3-3A). MR-proADM levels averaged approximately 0.35 nmol/L, which is nearly equivalent to the mean MR-proADM concentration of 0.33 nmol/L across the general population.[22] Subsequently, and consistent with higher levels of AM in eutopic endometrium of women with endometriosis, we found that circulating plasma levels of MR-proADM were higher in women with endometriosis compared to healthy controls (Figure 3-3B). However, MR-proADM levels did not vary by stage of disease (Figure 3-3C).

We then asked whether the elevated levels of MR-proADM were corrected by surgical resection of ectopic endometrial lesions. Analyzing MR-proADM concentrations immediately after surgery and three months after surgery, we determined that elevated MR-proADM levels persisted through the post-surgical period (Figure 3-3D). Finally, we assessed whether MR-proADM concentrations before, after, and three months after surgery

correlated with whether participants were able to become pregnant. We did not find any difference in MR-proADM levels between women with endometriosis who became pregnant and those who did not (Figure 3-3E and F).

Discussion

In this study, we have presented evidence for *Adm* as a potential STAT3 target in the uterus and investigated MR-proADM as a potential biomarker of endometriosis. Prior evidence for a STAT3-*Adm* axis is two-fold: first, pSTAT3 and *Adm* levels are positively correlated in breast cancer;[16] and second, activation of STAT3 by oncostatin M promotes *Adm* transcription in astroglioma cells.[17] However, to our knowledge, this is the first direct evidence for a STAT3-*Adm* axis in the uterus. This finding is not surprising, as pSTAT3 and AM are co-localized in the uterus during early pregnancy.[28,29] Furthermore, reduction of STAT3 or AM in the uterus causes problems with endometrial receptivity and implantation, potentially due to defects in epithelial cell polarity, among other causes.[28-31] It will be the subject of future studies to determine whether STAT3 causally upregulates *Adm* expression by directly binding active sites in the *Adm* promoter in uterine cells and if this interaction is critical for reproductive physiology and pathophysiology, specifically in the context of endometriosis.

We then asked whether AM, like pSTAT3, was upregulated in the eutopic endometrium of women with endometriosis, finding more AM in all epithelial and stromal compartments of the uterus. The increased expression is consistent with previously demonstrated elevated pSTAT3 and HIF1A abundance in the eutopic endometrium of women with endometriosis.[8] However, the expression of AM in ectopic lesions remains unknown.

Because MR-proADM is a stable surrogate for AM peptide in circulating plasma, we compared levels of MR-proADM in women with and without endometriosis. This is the first

study, to our knowledge, to examine levels of MR-proADM across the menstrual cycle. As the MR-proADM assay was developed just over a decade ago, reference intervals in different physiological conditions and disease states are just emerging.[32] Here, we contribute evidence for stable levels of MR-proADM across all stages of the menstrual cycle in healthy women.

Comparison of MR-proADM concentrations in women with and without endometriosis led to our primary finding of elevated MR-proADM in women with endometriosis. We posit several explanations for the finding: First, endometriosis is well-understood to be an estrogenic disease, and *Adm* is transcriptionally regulated by estrogen.[33,34] Second, endometriosis is an inflammatory condition with consequences for fertility, and AM is an anti-inflammatory peptide commonly upregulated in response to inflammation.[35,36] Endometriosis is also associated with an elevated risk of epithelial ovarian cancer, and AM has been shown to promoter angiogenesis in this subtype of ovarian cancer.[37-39] Therefore, our finding of elevated MR-proADM in women with endometriosis is consistent with associations common to both endometriosis and AM.

However, MR-proADM levels were not correlated with disease stage or corrected by surgical resection of ectopic lesions. Endometriosis staging determined primarily by the location and extent of ectopic lesions, and surgical resection aims to remove these lesions. Therefore, if ectopic lesions were the primary source of circulating MR-proADM, we would expect MR-proADM levels to be positively correlated with disease stage and to be corrected by surgery. While the expression of AM in ectopic lesions is not examined in this study, together, these data suggest that ectopic lesions are not the primary source of circulating MR-proADM.

MR-proADM levels were not predictive of fertility outcome in women with endometriosis, which is associated with subfertility or infertility, in women who underwent surgery. However, studies by our group and others demonstrate that levels of both

maternal- and fetal-derived AM are important for fertility and pregnancy.[19,20] It appears that AM levels are carefully titrated in normal physiology; for example, while *Adm*$^{-/-}$ mice are embryonic lethal, *Adm*$^{hi/hi}$ mice, which overexpress AM, develop hyperplastic hearts during development.[40,41] Therefore, it is becoming clear that concentrations of AM at either extreme can be detrimental to normal physiology.

In summary, we present evidence for a potential STAT3-*Adm* interaction in the uterus; for upregulation of AM in the eutopic endometrium of women with endometriosis; and for higher circulating levels of MR-proADM in endometriosis. Whether elevated MR-proADM in endometriosis is secondary to the estrogenic and inflammatory properties of endometriosis or an inciting pathogenic factor will be the subject of future study.

TABLES

	Control (n=19)	Endometriosis (n=28)
Age (years)	26.2 ± 4.4 (20-33)	32.9 ± 4.9 (23-41)
BMI	22.4 ± 2.9 (18.3-28.2)	23.7 ± 4.7 (18.7-42.5)
Gravidity at biopsy	0 (0-3)	0 (0-4)
Race		
Caucasian	9	26
African-American	7	0
Asian	3	1
Multiple	0	1

Age and BMI are presented as mean ± standard deviation (range). Gravidity is presented as median (range).

Table 3-1. Clinical characteristics of study participants donating plasma for MR-proADM analysis.

FIGURES

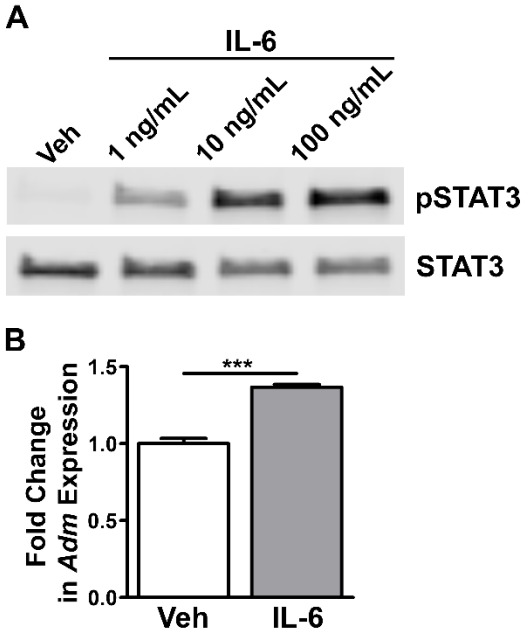

Figure 3-1. IL-6 activation of STAT3 upregulates *Adm* expression in Ishikawa cells. A, Western blot analysis of pSTAT3 expression in Ishikawa cells following a 15 minute treatment of varying doses of IL-6. n=1 biological replicate in Ishikawa cells, although the blot was also performed in primary human endometrial stromal cells as well as in a trophoblast cell line (HTR-8/SVneos) with nearly identical results. B, qRT-PCR analysis of *Adm* expression in Ishikawa cells after a 15 minute treatment of 100 ng/mL IL-6. ***p<0.001, unpaired t-test.

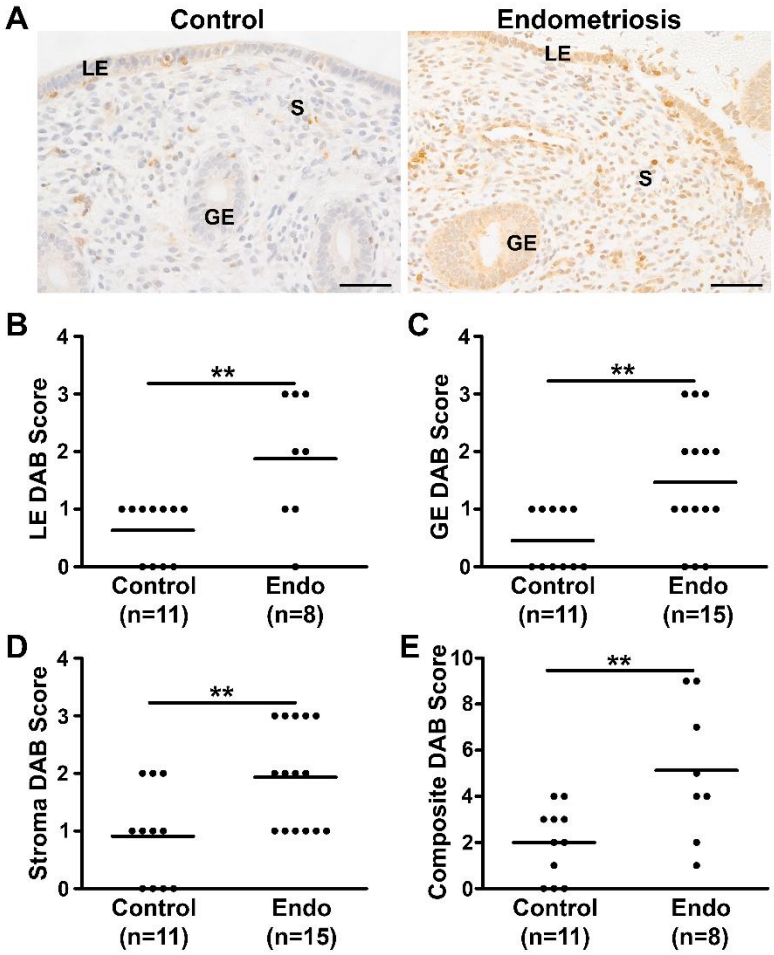

Figure 3-2. Immunohistochemistry for AM in endometrial biopsies reveals elevated levels of AM in women with endometriosis. A, Representative images of immunohistochemistry for AM in eutopic endometrial biopsies from women with endometriosis and from healthy controls. GE, glandular epithelium; LE, luminal epithelium; S, stroma. B-D, Semi-quantitative analysis of AM staining in luminal epithelium (B), glandular epithelium (C), and stroma (D) of endometrial biopsies. DAB score reflects no (0), weak (1), moderate (2), or strong (3) staining. **p<0.01, Mann-Whitney test. E, Composite DAB score calculated as the sum of the compartmental DAB scores depicted in B-D. Luminal epithelium was not present in all endometrial biopsies from women with endometriosis, therefore LE DAB score and composite DAB score were unable to be calculated in all biopsies. Each dot represents an individual patient. **p<0.01, unpaired t-test.

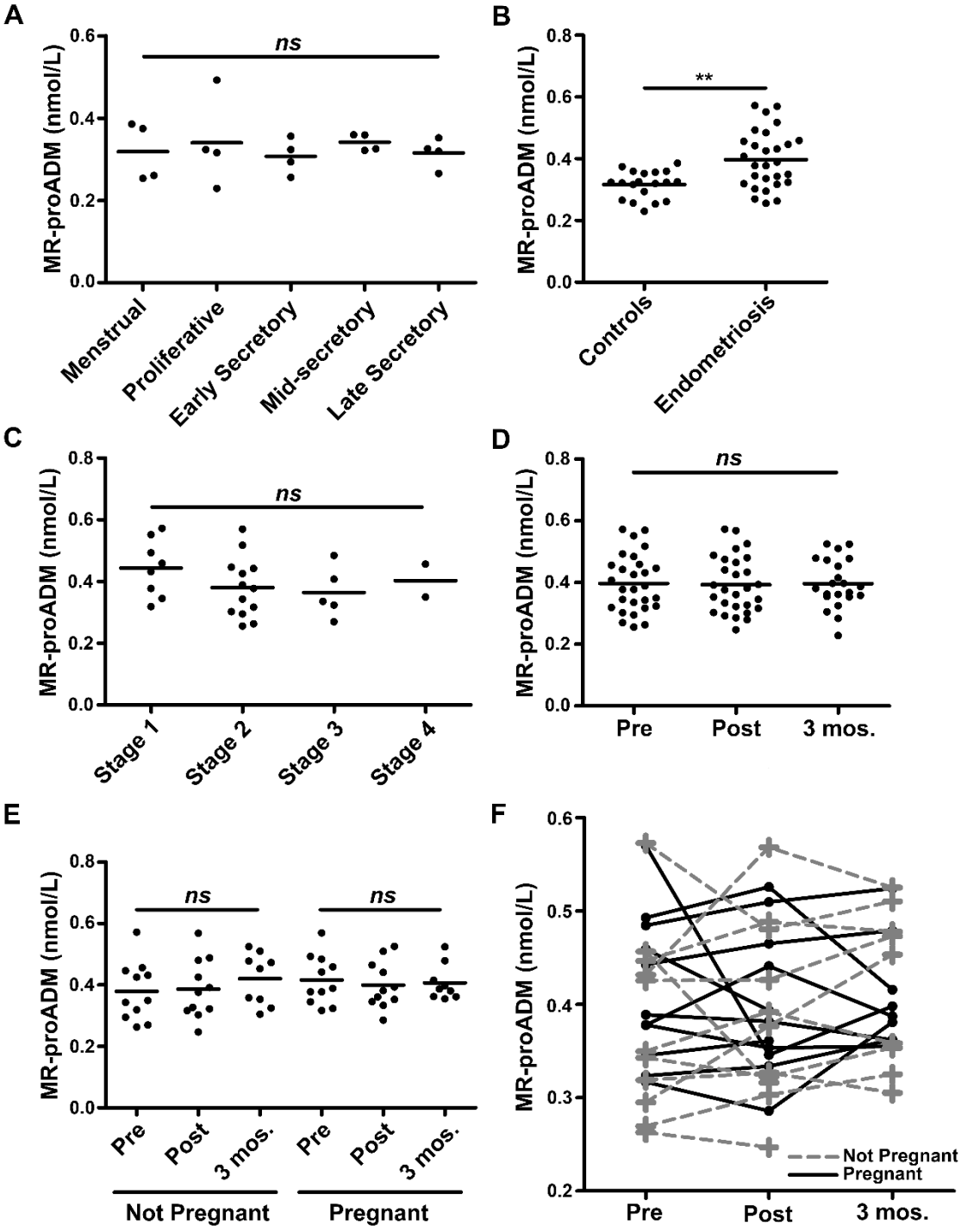

Figure 3-3. MR-proADM plasma concentrations are elevated in women with endometriosis but not correlated with disease stage, surgical status, or pregnancy outcome. A, MR-proADM concentrations in plasma from healthy women across all stages of the menstrual cycle. *ns*, not significant, Kruskal-Wallis. B, MR-proADM concentrations in healthy controls and women with endometriosis. **p<0.05, unpaired t-test. C, MR-proADM concentrations in women with endometriosis binned by disease stage and severity. ns, not

significant, Kruskal-Wallis test. D, MR-proADM concentrations in women with endometriosis before, immediately after, and three months after surgery. ns, not significant, Kruskal-Wallis test. E, MR-proADM concentrations in women with endometriosis pre-, post-, and three months post-surgery, binned by those who became pregnant and those who did not. ns, not significant, Kruskal-Wallis test. F, Individual, patient-level MR-proADM concentrations over time spanning the surgical period.

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Chapter 4: Conclusions and Future Directions

Summary of Results

Our basic science studies using genetic loss-of-function and pharmacologic gain-of-function mouse models, presented in **Chapter 2**, demonstrate that administration of AM peptide directly into the uterus prior to blastocyst transfer improves implantation success and inter-embryonic spacing. Examination of the uterine luminal epithelium in this AM-supplemented *in vitro* fertilization (IVF) model revealed morphological changes believed to be associated with endometrial receptivity and therefore fertility. Namely, we observed a greater number of pinopodes, plasma membrane extravasations of the epithelium projecting into the lumen of the uterus, which were bigger in size and covered a larger percentage of the epithelial surface compared to the pinopodes in vehicle-treated animals. As pinopodes have been associated with pinocytosis in rats, we then took advantage of a uterine luminal epithelial-like cell line to test whether AM affected movement of ions and water across the plasma membrane. We found that AM treatment improved plasma membrane permeability, accelerating water transport across the apical membrane unaccompanied by changes in ion transport.

We also examined changes in junctional proteins in the epithelial and stromal compartments of the uterus with varying dosages of AM peptide. Compared to wild type animals, female mice haploinsufficient for *Adm* demonstrated aberrant localization of CLDN1, a tight junction protein previously associated with fertility, in the uterine luminal epithelium. In stroma, we found that AM enhanced gap junction-mediated communication,

which could potentially explain the decreased functionality of the PDZ barrier observed in heterozygous animals.

These junctional phenotypes of loss- and gain-of-AM-function *in vivo* mouse models, together with a precedent for a STAT3-*Adm* interaction,[1] prompted us to test whether AM might be a downstream target of STAT3. Indeed, our *in vitro* studies in **Chapter 3** using Ishikawa cells, a uterine luminal epithelial-like cell line, demonstrate that activation of STAT3 via IL-6 upregulates *Adm* mRNA expression. This data provides a first glimpse into a potential STAT3-*Adm* interaction in the uterus.

Finally, after demonstrating clear benefit for AM supplementation during implantation and establishing preliminary evidence for *Adm* as a downstream target of a signaling pathway essential for fertility, we turned to available clinical samples from women with endometriosis to ask whether AM levels were changed in a disease associated with infertility. First, we found elevated AM by immunohistochemistry in eutopic endometrial biopsies from women with endometriosis compared to biopsies from healthy donors. We then took advantage of a commercial assay for MR-proADM, a precursor of the mature AM peptide, as a circulating biomarker in plasma. Consistent with the biopsy data, we found higher circulating levels of MR-proADM in women with endometriosis. However, MR-proADM concentrations were not correlated with disease stage nor corrected by surgery or predictive of pregnancy outcome.

Future Directions

At the Molecular Level – Regulation of AM Signaling in the Uterus

In **Chapter 3**, we suggest that activation of STAT3 by IL-6 upregulates *Adm* expression in uterine cells, implicating *Adm* as a downstream target of STAT3. We chose IL-6 as an activator of STAT3 in our *in vitro* experiments because aberrant IL-6-STAT3 signaling has been associated with endometriosis.[2-5] However, in the future, we would be

remiss not to consider other activators of STAT3, of which there are many. It would be especially important to test LIF as an activator of STAT3, as the LIF-STAT3 axis is a key signaling pathway supporting endometrial receptivity and female fertility. Regardless of the ligand/receptor combination that activates STAT3 and leads to *Adm* upregulation, we would then begin our search for active STAT3 response elements (STREs) in the *Adm* promoter. One published study presents two different STREs in the *Adm* promoter, but details on the discovery of these two sites are lacking.[1] Therefore, we could interrogate the *Adm* promoter for STAT3 binding motifs and then interrogate these potential STREs using luciferase assays and chromatin immunoprecipitation (ChIP). In the context of these assays, we could then interfere with STAT3 signaling by lentiviral or pharmacological knockdown of STAT3 or by generating point mutations in candidate STREs.

One caveat to our *in vitro* studies in **Chapter 3** is the use of Ishikawa cells as a model for receptive uterine luminal epithelium. Ishikawa cells often generate skepticism because they were derived from an endometrial adenocarcinoma.[6] However, commercially available cell lines that are better models of receptive epithelium do not exist.[7] We would be better served by collecting primary epithelial cells from either mice or humans, but the collection and subsequent use of these cells in cell culture studies like luciferase assays and ChIP as discussed above are technically cumbersome.

Given the dynamic environment of the uterus during early pregnancy and implantation, it would be worth considering whether the regulation of AM signaling is different in the epithelial and stromal compartments of the uterus. In contrast to primary epithelial cells, primary human endometrial stromal cells (hESCs) are available in our laboratory and easy to handle in cell culture. *In vivo*, we could take advantage of available *Cre* lines that delete in the epithelium (*Ltf-iCre*) and stroma (*Amhr2-Cre*) of the uterus.[8,9] We could cross these *Cre* lines to the floxed STAT3 line to delete STAT3 in the epithelium and stroma and then to the *Rpl22*$^{tm1.1Psam}$ (RiboTag) line, enabling us to analyze compartment-

specific mRNA based on which promoter is driving *Cre* expression.[10] If STAT3 upregulates *Adm* expression, we would expect STAT3 excision by *Cre* recombinase to downregulate *Adm* mRNA. This may be true in one or both compartments of the uterus and may be different depending on the day of pregnancy studied. Ultimately, if we established STAT3 as a transcriptional activator of *Adm*, we would have identified an additional downstream target of STAT3 in the uterus and an additional regulator of *Adm* expression, third to estrogen and hypoxia via HIF1-α.[11-13]

At the Cellular Level – Epithelial Cell Polarity and Ultrastructure

In **Chapter 2**, we begin to explore the role of cell junction proteins in the endometrium during implantation. In epithelium, we examined CLDN1, a tight junction protein, in the context of *Adm* haploinsufficiency. In stroma, we examined Cx43, a gap junction protein, in the context of exogenous AM supplementation. However, there are many other junctional proteins expressed in the epithelium and stroma. Several of these have been implicated in fertility as it is becoming clearer that loss of epithelial cell polarity is an important prerequisite for healthy embryo implantation.[14,15] It would be important to study other junctional proteins in the epithelium and stroma throughout early pregnancy in *Adm*$^{+/-}$ animals as well as in wild type AM-treated animals. Examples of other junctional proteins we could study include additional claudins, E-cadherin, α-catenin, β-catenin, ZO-1, and ZO-2 based on previously published studies on the expression of these proteins in the endometrium and in the early implantation site.[16-18]

Perhaps the most visually compelling images from our studies are the scanning electron micrographs of pinopodes presented in **Chapter 2**. Currently, scanning electron microscopy (SEM) is the best – and essentially the only – way to visualize pinopodes, limiting the questions that we are able to ask. Immunogold labeling in the context of SEM is possible and has been done by one group to study the expression of galectin-9 in

pinopodes.[19] However, immunogold labeling cannot facilitate colocalization studies, as only one protein can be labeled at a time. Therefore, the application of imaging modalities other than SEM, such as whole mount immunofluorescence, to the study of pinopodes would be highly desirable and informative. Basic yet important questions about pinopodes that could be answered using confocal fluorescent microscopy include: Are they different than cell blebs found in other epithelial layers? What proteins are expressed on the surface of pinopodes? What proteins, if any, are expressed within these structures?

Once we answer these questions and identify reliable markers of pinopodes, we may be able to use these findings to help us establish an *in vitro* kinetic model to interrogate pinopode function. One group claims to have visualized pinopodes by SEM in an *in vitro* co-culture system consisting of Ishikawa cells and hESCs.[20] However, as discussed previously, the use of Ishikawa cells presents its own challenges, and co-culture of primary human luminal epithelial cells and hESCs may be a better alternative. Establishment of an *in vitro* system that generates pinopodes could then be used to determine pinopode function. The hypothesized pinocytotic function of these structures led to the term “pinopode.” However, the few published studies on this pinocytotic function have come to different conclusions based on the species in question. For example, there is support for pinopode pinocytosis in rats but not in humans.[21-24] Whether pinopodes demonstrate pinocytosis in the mouse is unknown. With an *in vitro* kinetic model in hand, we may be able to address this question. We could also investigate the interactions or lack thereof between pinopodes and the implanting blastocyst, as it is unknown whether pinopodes facilitate or obstruct initial contact of the blastocyst with the epithelium.

Both *in vitro* and *in vivo* studies on pinopodes are limited by the unavailability of a standard way to pharmacologically or genetically eliminate pinopodes. Several studies point to estradiol; RU486, a progesterone inhibitor; testosterone; and metoclopramide, a dopamine receptor antagonist, as pharmacological agents that decrease pinopode

expression.[25-28] Therefore, we are presented with several avenues to pursue in the establishment of a negative control for our *in vitro* and *in vivo* studies on pinopodes. Of these three options, we are likely to prioritize RU486 given the positive correlation between serum progesterone concentration and pinopode expression in humans.[29] If administration of RU486 to a pseudopregnant or pregnant mouse is able to eliminate pinopode expression, these animals could serve as negative controls for our planned whole mount immunofluorescence studies.

At the Organ Level – Endometrial Physiology during Implantation

An especially interesting finding presented in **Chapter 2** is that AM enhances the permeability of the Ishikawa cell apical membrane, leading to accelerated water transport. Water transport plays an important but underappreciated role in the endometrium, as it facilitates appropriate luminal fluid viscosity and closure of the lumen around the embryo at the time of implantation. There are many studies characterizing aquaporin expression in the endometrium but only one study linking aquaporins and AM: One study found a positive correlation between plasma AM levels and urinary aquaporin 2 levels in human patients under general anesthesia, but whether there is a link between aquaporins and AM in the endometrium is to be determined.[30]

Similarly, there is a sizeable body of literature on ion channels, including cystic fibrosis transmembrane conductance regulator (CFTR), epithelial sodium channel (ENaC), Ca^{2+} channels, and K^{+} channels and their importance in endometrial receptivity and implantation.[31] In our Ussing chamber studies presented in **Chapter 2**, we did not find changes in Ishikawa cell short-circuit current or transepithelial resistance after addition of a series of ion channel modulators following AM pre-treatment. However, these studies were performed in Ishikawa cells, so we could consider repeating these studies in primary human endometrial epithelial cells. *In vivo*, we could interrogate links between AM and aquaporins

and ion channels by assessing luminal fluid content as well as aquaporin and ion channel mRNA expression in several different mouse models: 1) our AM-supplemented blastocyst transfer model; 2) our *Adm* haploinsufficient model of subfertility; and/or 3) deletion of the AM receptor and therefore AM signaling using endometrial compartment-specific *Cre* lines.

Another piece of data presented in **Chapter 2** that supports a link between AM and aquaporins and ion channels is the finding that administration of AM prior to blastocyst transfer equalized spacing between embryos at e6.5. While embryo spacing in the uterus is incompletely understood, there are many hypothesized determinants of spacing – including water and ion channels function and consequent changes in luminal fluid.[32] One potentially high-yield experiment would be to perform our AM-supplemented blastocyst transfer protocol in *Ltf-iCre;Rpl22*$^{tm1.1Psam}$ (RiboTag) pseudopregnant recipients and then perform a microarray on RiboTag-tagged mRNA, which would be specific to luminal epithelium. It is possible that this experiment would identify changes in aquaporins and/or ion channels with AM treatment – or other, unexpected pathways – that could explain the enhanced implantation rate and equalized spacing between embryos.

At the Organism Level – Rescue of Alternative Models of Subfertility and Infertility

Our primary finding of enhanced implantation success after pre-implantation administration of AM was obtained in wild type CD1 mice. It would be interesting to test whether we could rescue our *Adm* haploinsufficient mouse model of subfertility with AM. We could also perform our AM-supplemented blastocyst transfer protocol in wild type mice of another strain with below average fertility – in either commercially available strains or several strains from UNC's Collaborative Cross. Alternatively, we could antagonize endometrial receptivity pharmacologically and then attempt to rescue with AM. For example, systemic morphine administration has been shown to interfere with endometrial receptivity.[33] We attempted to co-treat with complement factor H (CFH), the binding partner of circulating

AM peptide, in order to bolster the AM effect but surprisingly found that co-treatment with CFH was antagonistic to implantation. A couple of hemodynamic studies have found that co-treatment with a neural endopeptidase inhibitor enhances the efficacy of AM, so we could also considering incorporating this treatment scheme into our blastocyst transfer protocol.[34,35]

Finally, we could look beyond the peri-implantation period at mid-gestation and early postnatal development to ensure that embryos are not adversely affected by the pre-implantation administration of AM. For example, we could compare litter sizes between vehicle- and AM-treated animals at e13.5 and at birth and embryo and placenta weights at each of these time points. One potential concern is embryonic cardiac hyperplasia as demonstrated by *Adm*$^{hi/hi}$ embryos.[36] However, these embryos are constitutively exposed to high levels of AM, whereas the embryos in our blastocyst transfer model are only transiently exposed. These studies would be especially important to perform before clinical usage of AM in the context of assisted reproductive technology becomes a possibility.

In the Clinic – AM as Therapeutic for Infertility

Ultimately, we believe that administration of AM directly into the uterus could improve the clinical pregnancy rate following assisted reproductive technology like intrauterine insemination (IUI) or IVF. To this end, first-pass experiments in the clinic could address the safety of AM treatment in humans. For example, we could infuse AM directly into the uterus for a short time while monitoring patients for safety, paying particular attention to any hemodynamic abnormalities. Because the delivery of AM – a potent vasodilator – is localized and not systemic, we are optimistic that this delivery route would avoid hypotensive episodes. We could then obtain endometrial biopsies for analysis of pinopodes by SEM in an attempt to recapitulate our mouse findings in humans. The correlation between pinopode expression and endometrial receptivity is debated, as both *Lif*$^{-/-}$ and

Hoxa10$^{-/-}$ mice are infertile but have pinopodes,[37] but a clinical study just published this year maintains a link between pinopodes and fertility.[38]

In the past several years, an endometrial receptivity array (ERA) has been developed to characterize the gene signature of the receptive endometrium and to assist with the diagnosis of endometrial-factor infertility.[39,40] Therefore, it would be interesting to collect RNA from endometrial biopsies and compare ERA outputs between vehicle- and AM-infused uteri, which we could then compare to the effects of endometrial scratching and G-CSF administration, both of which have been shown to enhance clinical pregnancy rates in assisted reproductive technology.[41,42]

Finally, we examined MR-proADM as a biomarker of endometriosis, which is a co-morbid with subfertility and infertility, finding elevated levels of MR-proADM in plasma from women with endometriosis compared to healthy controls. Whether this elevation is because AM is involved in the pathophysiology of endometriosis or secondary to the estrogenic and inflammatory environment of endometriosis is unknown. If we wish to pursue AM as a therapy for infertility, perhaps it would be better to analyze MR-proADM concentrations in women with endometrial-factor infertility and recurrent implantation failure (RIF). Based on our studies on AM and female fertility in mice, we would predict that women with RIF would have lower levels of MR-proADM than healthy women. Women with RIF and low MR-proADM would then represent a germane patient population in which we could pilot AM therapy to improve clinical pregnancy and live birth rates following IUI and IVF.

Concluding Remarks

In **Part I**, we have followed up on the Caron laboratory's *Adm* haploinsufficiency and subfertility studies from a decade ago.[43,44] Specifically, we highlight potential mechanisms of action of AM in the epithelial and stromal compartments of the endometrium supporting

implantation and early pregnancy. We also conclude that administration of AM directly into the uterus prior to blastocyst transfer improves embryo implantation success, providing a foundational basis for using AM as a clinical therapy for infertility, likely in combination with assisted reproductive technology like IUI and IVF. The stage is set for many experiments in both the laboratory and the clinic that will inform our efforts to use AM to enhance the live birth rates following these procedures. Finally, based on our MR-proADM biomarker studies, we also suggest that AM may be involved in the pathophysiology of endometriosis, a disease associated with subfertility and infertility, though further study is necessary to determine whether this is cause or consequence of the endometriosis disease process.

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PART II – MAINTENANCE OF PREGNANCY

Chapter 5: Uterine Natural Killer Cells as Modulators of the Maternal-Fetal Vasculature[1]

Overview

Precise and local control of the innate immune system within the placenta is an essential component for achieving a normal and healthy pregnancy. One of the most abundant immune cells of the placenta is a subpopulation of natural killer (NK) cells that profusely populates the uterine decidua during early pregnancy. Uterine NK (uNK) cells and trophoblast cells of the placenta communicate both directly and indirectly to contribute to the critical process of spiral artery remodeling. Here, we discuss recent findings that expand our knowledge of uNK cell-trophoblast cell crosstalk and the important role it plays in the maternal vascular adaptation to pregnancy.

Introduction

Natural killer (NK) cells are lymphocytes belonging to the innate immune system that attack virally-infected cells and tumor cells via exocytosis of perforin- and granzyme-containing granules. In females, there are two populations of NK cells: peripheral blood (pb) NK cells and decidual (d) or uterine (u) NK cells. Both NK cell populations are capable of

[1]Reprinted with permission from: Matson BC & Caron KM. Uterine natural killer cells as modulators of the maternal-fetal vasculature. *Int J Dev Biol*. 2014;58:199-204.

cytotoxicity and cytokine secretion. However, pbNK cells are primarily lytic cells, while uNK cells are primarily cytokine and chemokine producers.

A dramatic expansion of uNK cells occurs during early pregnancy, populating two adjacent areas of the implantation site, the decidua basalis (DB) and the mesometrial lymphoid aggregate of pregnancy (MLAp). Proliferation continues until mid-pregnancy, at which point uNK cells comprise up to 70% of immune cells present in the decidua, the progesterone-altered endometrium of the uterus that supports the conceptus.[1] uNK cell population size then declines until the end of pregnancy.

Molecular characterization of uNK cells has led to the identification of subpopulations of uNK cells in the decidua. Mouse uNK cells, previously identified by periodic acid Schiff (PAS) staining, are also currently identified by Dolichos biflorus agglutinin (DBA) lectin staining.[2] PAS and DBA lectin staining defines two subpopulations of uNK cells: $PAS^{+}DBA^{-}$ and $PAS^{+}DBA^{+}$ cells, which exhibit different gene expression profiles.[3,4] While PAS is a pan-uNK cell marker, DBA lectin detects the subpopulation of cells that expands during pregnancy, as 90% of uNK cells at mid-gestation are DBA^{+}.[4]

In contrast to the mouse, human uNK cells are identified by their CD56 CD16 signature. The vast majority of uNK cells are $CD56^{bright}CD16^{-}$, and pbNK cells are typically $CD56^{dim}CD16^{+}$. Interestingly, while both subpopulations are present in the decidua, the proportion of these two subsets can shift to favor cytotoxic $CD56^{dim}CD16^{+}$ cells in the presence of infectious agents like cytomegalovirus[5] and *Toxoplasma gondii*,[6] both common intrauterine infections that cause severe birth defects.

Despite belonging to the immune system, the primary contributions of uNK cells to the developing pregnancy are not immune in nature. Rather, uNK cell-secreted cytokines and chemokines communicate with fetal trophoblast cells of the placenta.[7] These two cell types act in concert to remodel spiral arteries, conduits of blood from the uterus to the placental bed and growing fetus.[8] The importance of this process is stressed by the

association of insufficient spiral artery remodeling with several diseases of pregnancy, such as fetal growth restriction (FGR) and preeclampsia. Here, we briefly review aspects of uNK cell-trophoblast cell crosstalk and their role in spiral artery remodeling and the maintenance of pregnancy.

uNK Cell Differentiation

Little is known about uNK cell precursors and the source of the expanded uNK cell population during pregnancy. However, there is evidence to suggest that the majority of uNK cell precursors originate from outside the uterus. In the early 2000s, Croy and colleagues were unable to detect differentiated uNK cells in NK cell-deficient animals engrafted with parts of wild type uteri, suggesting that uNK cell precursors are extra-uterine.[9] More recently, analysis of changes in uNK cell surface markers during pregnancy suggest that uNK cells endogenous to the uterus decline between gestation day (gd) 0 and gd6, perhaps because their cytotoxicity would be lethal to the foreign conceptus.[10] Therefore, the endogenous uNK cell population may be less critical for the maintenance of pregnancy than uNK cells that differentiated from extra-uterine precursors.

Macrophage-derived interleukin-15 (IL-15) is a critical regulator of NK and uNK cell differentiation. It was first observed that the time course of IL-15 expression during pregnancy parallels that of uNK cell granule contents.[11] Soon thereafter, *IL-15*$^{-/-}$ animals were generated.[12] *IL-15*$^{-/-}$ females lack uNK cells, MLAps, and spiral artery remodeling despite birthing litters of average size but slightly below-average weight.[13,14] Recent microarray analysis comparing *IL-15*$^{-/-}$ and wild type animals did not detect differences in expression levels of genes involved in decidualization.[15] Therefore, the finding of below-average weight pups born to *IL-15*$^{-/-}$ dams cannot be explained by differences in decidualization and is likely due to the effect of IL-15 on uteroplacental circulation via stimulation of uNK cell differentiation.

Recent studies offer candidate regulators of IL-15 expression. For example, there is evidence that the transcription factor RUNX3 acts together with other transcription factors to promote IL-15 expression.[16] However, there are also potentially indirect regulators of IL-15 expression. For example, a conditional knockout (cKO) of bone morphogenic protein receptor 2 (*Bmpr2*) in the female reproductive system demonstrated decreased IL-15 expression.[17] As may be expected, *Bmpr2* cKOs lacked uNK cells at implantation sites and exhibited defects in placentation.

In another animal model of placental underperfusion, heme oxygenase-1 (HO-1) heterozygotes and knockouts also downregulated IL-15 expression and exhibited fewer uNK cells in the DB and MLAp.[18] Similar to *Bmpr2* cKOs, *Hmox1*$^{+/-}$ dams demonstrated intrauterine growth restriction (IUGR), suggesting poor uteroplacental circulation. Interestingly, treatment with CO, a byproduct of HO-1's heme metabolism, elevated uNK cell numbers, promoted spiral artery remodeling, and decreased the incidence of fetal death. However, CO treatment did not elevate IL-15 levels. In an attempt to explain why IL-15 is downregulated in *Hmox1*$^{+/-}$ and *Hmox1*$^{-/-}$ animals, the authors suggest that CO may affect the activity of macrophages, a demonstrated source of IL-15 in the pregnant uterus.[11] If true, this hypothesis could address the well-established, counterintuitive protection that smoking confers against preeclampsia. Altogether, these observations suggest potential indirect mechanisms of uNK cell differentiation via regulation of IL-15 expression.

uNK-Trophoblast Cell Interactions: KIRs and HLAs

Differentiated uNK cells express activating and inhibitory cell surface receptors. Stimulation of these subtypes by trophoblast-expressed ligands, for example, determines the degree of uNK cell activity. Interestingly, the proportions of activating and inhibitory receptors may shift in the presence of a foreign pathogen to modify uNK cell activity and promote cytotoxicity.[19] Many of these uNK cell surface receptors belong to the killer cell Ig-

like receptor (KIR) family. KIR A and B haplotypes preferentially express inhibitory and activating receptors, respectively, and bind to fetal trophoblast-expressed human leukocyte antigen C (HLA-C), a major histocompatibility complex (MHC) type I molecule.

Importantly, Moffett and colleagues demonstrated associations of certain KIR-HLA combinations – specifically KIR AA and HLA-C2 – with diseases of pregnancy like preeclampsia and miscarriage.[20,21] Binding of fetal HLA-C2 to the inhibitory receptor KIR2DL1 in these women may predispose to preeclampsia and other placental disorders via insufficient uNK cell activation. In contrast, KIR B women preferentially express uNK cell activating receptors. In these women, HLA-C2 likely binds to the activating receptor KIR2DS1, protecting women against these diseases by activating uNK cells and stimulating trophoblast invasion.[20,22] Recent evidence that KIR2DS1 stimulates secretion of cytokines like granulocyte macrophage colony-stimulating factor (GM-CSF) by uNK cells and trophoblast migration supports this paradigm.[23]

While HLA-C has attracted attention for its disease associations, trophoblast cells express HLAs other than HLA-C. Specifically, they also express HLA-E and HLA-G. HLA-E binds to CD94/NKG2A, an inhibitory receptor on uNK cells,[24] and HLA-G binds to leukocyte immunoglobulin-like receptors (LILRs) on uNK cells.[25] However, HLA-G also binds to a KIR – CD158d/KIR2DL4, which is expressed in endosomes, not on the uNK cell surface like other KIRs.[26] Binding of HLA-G to KIR2DL4 activates downstream pathways that confer a senescent phenotype on the uNK cell.[27] Supernatants from KIR2DL4-stimulated uNK cells enhance the permeability and angiogenic capacity of human ubilical vein endothelial cells (HUVECs). It is easy, therefore, to imagine a role for uNK cells in placental vascular remodeling via HLA-G stimulation of KIR2DL4.

Despite the attention paid to the consequences of HLA-KIR interactions, there is likely a role for non-KIR uNK cell receptors that bind to non-HLA ligands. Specifically, the aryl hydrocarbon receptor (*Ahr*) is expressed by DBA^- uNK cells and may be important for

the proliferation of this oft-ignored uNK cell subset.[28] While *Ahr*$^{-/-}$ implantation sites demonstrated wild type levels of total uNK cells, DBA+ cells were smaller, and DBA^{-} cells were fewer in number.[28] *Ahr*$^{-/-}$ animals also demonstrated insufficient spiral artery remodeling. Similarly, loss of natural cytotoxicity receptors (NCR), expressed by DBA^{+} cells, didn't affect total uNK cell numbers but impaired uNK cell maturation and spiral artery remodeling.[28] While the ligands for these receptors are unknown, they are clearly playing an important role in uNK cell maturation and activity, highlighting the importance of interactions outside the KIR-HLA axis.

uNK Cell Recruitment by Trophoblast-derived Factors

uNK cell-trophoblast cell crosstalk extends beyond contacts between cell surface proteins. For example, trophoblast-derived peptides of the calcitonin (CT)/calcitonin gene-related peptide (CGRP) family are involved in maintaining proper placental perfusion, possibly by communicating with uNK cells. The clearest link between a CGRP family member, uNK cells, and spiral artery remodeling is adrenomedullin (AM).

Levels of AM, an anti-inflammatory vasodilator, are physiologically elevated in normal pregnancy[29] but altered in adverse pregnancy outcomes.[30] Polymorphisms in the AM gene are associated with birth weight, glycemic regulation, and preeclampsia.[31] AM localizes to implantation sites and is expressed by the uterine epithelium and fetal trophoblast cells.[32] Fetal trophoblast cells also express the AM receptor, calcitonin receptor-like receptor (CLR).[33] Interestingly, *Adm*$^{+/-}$ females are less fertile due to diminished endometrial receptivity,[34] and pups born to *Adm*$^{+/-}$ females are more likely to demonstrate FGR.[32] Altogether, these data suggest a role for maternal AM in implantation and placentation.

However, *Adm*$^{-/-}$ embryos are more likely to exhibit FGR than *Adm*$^{+/-}$ or *Adm*$^{+/+}$ embryos, suggesting a role for fetal-derived AM in placentation. *Adm*$^{-/-}$ placentas demonstrate fewer uNK cells and retention of vascular smooth muscle cells lining spiral

arteries compared to *Adm*$^{+/+}$ placentas.[35] Concordantly, placentas from *Adm*$^{hi/hi}$ pregnant females, a gene-targeted animal model of AM overexpression, exhibit 30% more uNK cells than AM$^{+/+}$ placentas and upregulate cytokine, chemokine, and matrix metalloproteinase (MMP) expression. *In vitro*, uNK cell-conditioned media supplemented with AM promotes apoptosis of vascular smooth muscle cells, supporting the emerging role for uNK cells in spiral artery remodeling.[35] It remains to be seen whether AM-mediated uNK cell recruitment and activity is dosage-dependent.

Like AM, adrenomedullin 2 (AM2), also known as intermedin, is physiologically elevated during pregnancy.[36] Administration of an AM2 antagonist causes FGR, highlighting the importance of AM2 in a healthy pregnancy.[37] AM2 is expressed by trophoblast cells and stimulates their invasion via the mitogen-activated protein kinase (MAPK) signaling pathway.[38,39] AM2 also dose-dependently stimulates HLA-G expression in trophoblasts cells, possibly stimulating KIR2DL4 signaling on uNK cells that could then acquire a senescent phenotype and increase vascular permeability and angiogenesis.[27,38] It is appealing to test whether AM2 dosage correlates with uNK cell recruitment to the decidua and consequent spiral artery remodeling.

Other CGRP family members also appear to modulate the uteroplacental circulation. For example, CGRP affects blood pressure regulation at the maternal-fetal interface, and its levels are altered in pregnancy-induced hypertension and preeclampsia.[40-44] However, CGRP's effects on uNK cell recruitment and activity remain to be elucidated.

As may be expected, there are trophoblast-derived factors outside the CGRP family that communicate with uNK cells. For example, thrombopoietin (TPO) and its receptor, c-Mpl, are expressed by uNK cells and trophoblast cells and act in concert to stimulate the proliferation and migration of these cell types via the JAK/STAT pathway.[45] As we come to understand more about uNK cell-trophoblast cell crosstalk, we imagine that other

trophoblast-derived factors will come to light as important effectors of uNK cell recruitment and activation.

Placental Vascular Remodeling by uNK Cell-derived Factors

uNK cells generate an array of angiogenic growth factors, cytokines, and chemokines in different proportions at different times of pregnancy, suggesting a continuous and evolving role for uNK cells as pregnancy progresses. Here, we briefly discuss several examples of uNK cell-derived factors, acknowledging that there are many others we do not address.

Angiopoietin (Ang) 1 and 2, transforming growth factor β (TGF-β), and vascular endothelial growth factor (VEGF) are several examples of uNK cell-secreted angiogenic factors.[46] Placental growth factor (PlGF) belongs to the VEGF family and has emerged as a potential biomarker for preeclampsia, emphasizing the significance of these angiogenic factors in proper uteroplacental circulation.[47,48] VEGF-C also belongs to the VEGF family and may assume an additional role – protecting trophoblast cells from uNK cell cytotoxicity.[49] The concentration of these factors decreases in the decidua as pregnancy progresses, suggesting that they may be irrelevant after spiral artery remodeling concludes.[46]

Interferon-gamma (IFN-γ) is a uNK cell-secreted cytokine that has arguably attracted the most attention in the literature; it is necessary and sufficient for spiral artery remodeling.[50] IFN-γ inhibits trophoblast invasion by promoting apoptosis of trophoblast cells and altering protease levels, keeping trophoblast invasion in check.[46] Perhaps counterintuitively, uNK cells from women with preeclampsia secrete less IFN-γ than uNK cells from normotensive controls.[51]

uNK cells also produce a variety of chemokines, including interleukin-8 (IL-8) and interferon-inducible protein-10 (IP-10). Receptors for these two chemokines, CXCR1 and

CXCR3, respectively, are expressed on the trophoblast cell surface, substantiating uNK cells' candidacy as potent regulators of trophoblast invasion.[7] However, IL-8 and IP-10 presence in uNK cell-conditioned media (CM) isn't different between pregnancies with normal and high uterine artery Doppler resistance indices.[52] To generate uNK cell CM, the authors plated equal densities of uNK cells from the different pregnancies, which could explain this finding. uNK cell chemokine secretion could very well be equivalent cell to cell, but the two types of pregnancies may have different population sizes of uNK cells. CM from these pregnancies did differ in extracellular signal-regulated kinase (ERK) and Akt pathway activation, which are critical for trophoblast invasion. Therefore, it is possible that uNK cells of these two types of pregnancies differentially express chemokines other than the ones examined in this study.

Finally, uNK cells are also sources of MMPs such as MMP-2.[53]The previously mentioned peptide hormone AM stimulates MMP-9 secretion from uNK cells, triggering spiral artery smooth muscle cell apoptosis.[35] Altogether, this orchestra of signaling molecules coordinates the complex process of spiral artery remodeling to maintain a healthy pregnancy. Intriguingly, angiogenic growth factors and cytokines decrease in concentration when uNK cells and trophoblast cells are co-cultured, though the cell type from which these factors are derived in this co-culture is uncertain.[54] No doubt additional signals will be identified that will further our understanding of uNK cell-trophoblast interactions.

Concluding Remarks

In summary, the overall immune milieu of the placenta is an important determinant of the health and success of a pregnancy.[55,56] Perturbations in this complex environment by lipopolysaccharide-induced inflammation, for example, can cause abnormal placental vascular remodeling and phenotypes resembling FGR and preeclampsia.[57] uNK cells dominate this immune landscape during early pregnancy and are important modulators of

the maternal-fetal vasculature. Generalized inflammatory changes triggered by obesity, for example, can cause under-recruitment of uNK cells to the decidua, which could explain why obesity elevates a patient's risk of pregnancy complications.[58]

Certainly, there are other determinants of uNK cell density in the decidua during early pregnancy not discussed here, such as decidual cell-derived cytokines.[59] However, it is likely that there are important effectors of spiral artery remodeling other than uNK cells;[60] several studies suggest that uNK cells may be important only during a small time frame of this process. Ultimately, furthering our understanding of uNK-trophoblast cell interactions and their role in placental vascular remodeling will shed light on placentation disorders like FGR and preeclampsia and may potentially reveal new treatment modalities for these diseases.

FIGURES

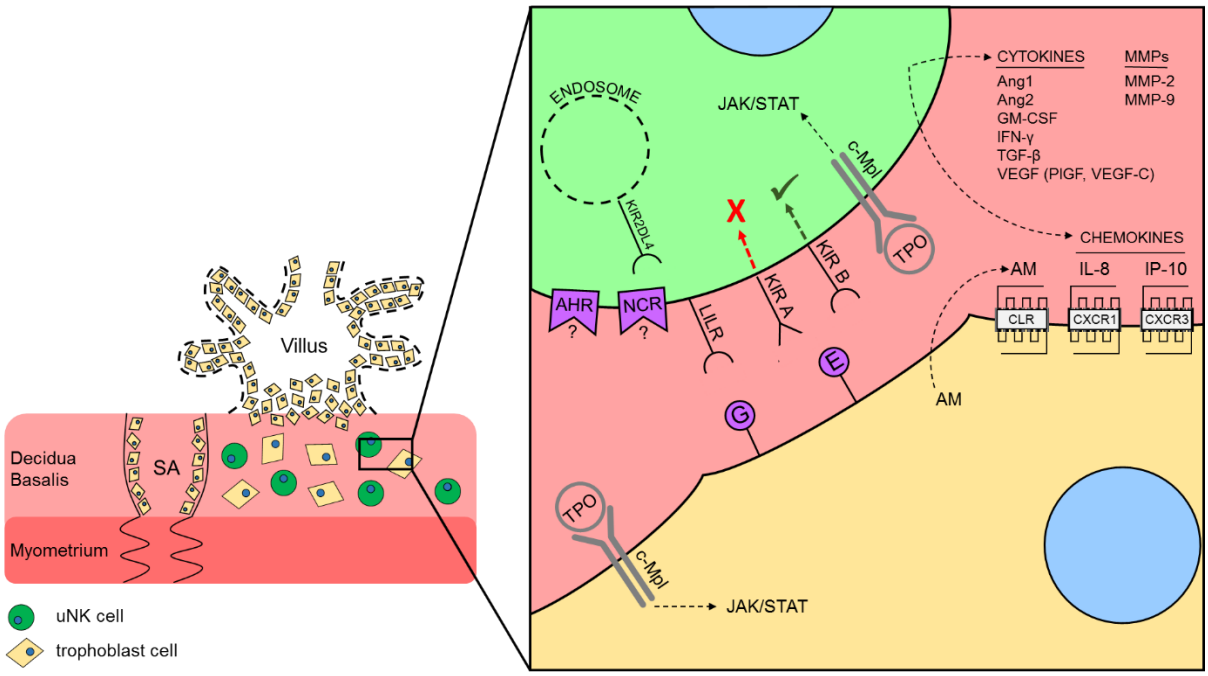

Figure 5-1. A combination of direct and indirect interactions between uterine natural killer cells (uNKs) and trophoblast cells influences spiral artery remodeling.

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Chapter 6: Adrenomedullin and Endocrine Control of Immune Cells during Pregnancy[1]

Overview

The immunology of pregnancy is complex and incompletely understood. Aberrant immune activity in the decidua and in the placenta is believed to play a role in diseases of pregnancy, such as infertility, miscarriage, fetal growth restriction, and preeclampsia. Here, we briefly review the endocrine control of uterine natural killer cell populations and their functions by the peptide hormone adrenomedullin. Studies in genetic animal models have revealed the critical importance of adrenomedullin dosage at the maternal-fetal interface, with cells from both the maternal and fetal compartments contributing to essential aspects underlying appropriate endometrial receptivity, implantation, and vascular remodeling of spiral arteries. These basic insights into the crosstalk between the endocrine and immune systems within the maternal-fetal interface may ultimately translate to a better understanding of the functions and consequences of dysregulated adrenomedullin levels in clinically complicated pregnancies.

Introduction

Pregnancy presents a mysterious immunological paradox that is permitted by the complex, unique immunology of the maternal-fetal interface. We are only beginning to

[1]Reprinted in part with permission from: Matson BC & Caron KM. Adrenomedullin and endocrine control of immune cells during pregnancy. *Cell Mol Immunol*. 2014;11:456-459.

understand how this very specialized immune “subsystem” differs from the systemic immune system and thus effectively protects the fetus from maternal rejection. For example, the sizable uterine natural killer (uNK) cell and macrophage populations found during early pregnancy are distinctive in their cell surface markers and functions compared to their peripheral counterparts.[1,2] Understanding the activity and control of these immune cell types will shed light on this immunological paradox and possibly inform the pathophysiology of complications of pregnancy.

During the past decade, numerous studies have characterized critical roles for the peptide hormone adrenomedullin (*Adm* gene; AM protein) in the establishment and maintenance of a healthy pregnancy. Here, we discuss the effects of AM on placentation, concentrating on the control of the uNK cell population and its subsequent involvement in the process of spiral artery remodeling – a necessary process for the maternal vascular adaptation to pregnancy. Importantly, studies addressing the link between AM and uNK cells exemplify an immunological basis for preeclampsia that can be strongly modulated by the maternal and fetal endocrine systems.

AM and Immune-based Placental Vascular Pathologies

Examination of *Adm*$^{-/-}$ mouse placentas and their vascular abnormalities revealed a direct link between fetal-derived AM and placental immunology. Notably, the placenta-perfusing spiral arteries of *Adm*$^{-/-}$ placentas are abnormally invested with a thick layer of vascular smooth muscle cells at e13.5.[3] By contrast, in wild type placentas from neighboring littermates, these vascular smooth muscle cells have undergone apoptosis during the process of spiral artery remodeling, which transforms the spiral arteries into large, high-capacitance vessels associated with the migration of fetal trophoblast cells into the arteries.[4] Importantly, insufficient spiral artery remodeling has been implicated in the pathophysiology of preeclampsia, a hypertensive disease of pregnancy. Therefore, the preeclampsia-like

phenotypes of *Adm*$^{-/-}$ placentas recapitulate prior evidence of dysregulation of AM levels in complications of pregnancy.[5]

Because uNK cells are the largest population of decidua-specific immune cells and are established effectors of spiral artery remodeling, uNK cells were counted in *Adm*$^{-/-}$ mouse placentas and found to be fewer in number compared to wild type placentas.[3] This reduction in uNK cell content was not associated with apoptosis but rather with under-recruitment of uNK cells to the decidua. Ovarian transplants (*Adm*$^{+/-}$ ovaries carrying *Adm*-null germ cells were placed in wild type recipient females) further confirmed that the *Adm*$^{-/-}$ placental immune and vascular phenotypes were due to the loss of *fetal*-derived AM and independent of the genotype or dosage of *Adm* from the dam.[3] This conclusion highlights a critical function for fetal-derived AM as an essential signal to elicit changes to the maternal vasculature during pregnancy.[6]

AM Dose-dependent uNK Cell Recruitment and Signaling

Subsequent studies of a gene-targeted murine model of *Adm* overexpression (*Adm*^{hi} allele, which expresses *Adm* at levels three-fold higher than the wild type allele) determined that uNK cell recruitment to the decidua by AM is dosage-dependent.[3,7] Specifically, *Adm*$^{hi/hi}$ placentas demonstrated a 30% increase in uNK cells.[3] Because the uNK cells in these placentas were labeled with *Dolichos biflorus* agglutinin (DBA) lectin, we can conclude that the uNK cell population that was quantified is, in fact, the one that expands during pregnancy.[8] Debate continues about whether this uNK cell population is derived from extra-uterine precursors that home to the uterus and differentiate *in situ* early in pregnancy or whether uNK cell precursors mature outside the uterus and then migrate to the uterus due to hormonal cues like AM.

Given the active participation of uNK cells in spiral artery remodeling as well as the uNK cell and vascular phenotypes of *Adm*$^{-/-}$ and *Adm*$^{hi/hi}$ placentas,[9] it stood to reason that

AM could augment the effects of uNK cells on vascular smooth muscle cells. Indeed, treatment of primary mouse vascular smooth muscle cells with uNK cell-conditioned media caused changes in cell morphology and induced apoptosis;[3] these processes were enhanced when the uNK cell-conditioned media was supplemented with AM, suggesting that AM is important not only for the recruitment but also for the activation of uNK cells.[3]

uNK cells produce an array of cytokines and chemokines that engage these cells in a complex dialogue with trophoblast cells to execute spiral artery remodeling.[9,10] Therefore, it was expected that the dynamic fluctuations in uNK cell population size between *Adm*$^{-/-}$ and *Adm*$^{hi/hi}$ placentas would be mirrored by concomitant changes in the expression profile of these signaling molecules. Indeed, *Adm*$^{-/-}$ downregulated and *Adm*$^{hi/hi}$ upregulated Ccl7, Ccl17, Cxcl10, Xcl1, and TNF.[3] Concordantly, *in vitro* stimulation of isolated uNK cells by AM upregulated select signaling factors, including matrix metalloproteinase 9, which is involved in spiral artery smooth muscle cell apoptosis.[3] Given the diversity of angiogenic factors, growth factors, and other signaling molecules that are secreted by uNK cells, it is plausible that there are signaling cascades other than the ones already identified that are regulated by AM.

Future Directions

Collectively, these data support AM as a player in the pathophysiology of reproductive disorders like preeclampsia via control of the uNK cell population size and of trophoblast invasion into the uterine luminal epithelium and into uterine spiral arteries. While perturbations in the size of the uNK cell population have been implicated in a variety of human reproductive disorders,[11,12] it has also been found that women with larger cytotoxic CD56^{dim}CD16^{+} uNK cell populations are at a higher risk for infertility and recurrent pregnancy loss.[13] Therefore, not only the uNK cell population size is important, but also the delicate balance between the peripheral blood-like, cytotoxic CD56^{dim}CD16^{+} uNK cells and

the “true” $CD56^{bright}CD16^{-}$ cytokine- and chemokine-producing uNK cells.[13] As argued by several groups, it is premature to base clinical decisions on information about size or type of uNK cell populations in patients.[12,14]

It will be interesting to determine whether AM exerts endocrine control over other immune cell populations at the maternal-fetal interface, such as decidual macrophages. There is evidence that AM can confer a semi-mature phenotype to dendritic cells, which provide instructions to T cells, but this may be of little consequence to placental immunology given the paucity of dendritic cells in the decidua.[15] Of course, it is also possible that other CGRP family peptides affect uNK cell recruitment. Adrenomedullin 2, also known as intermedin, has been shown to stimulate trophoblast invasion and therefore may participate in assemblage of the uNK cell population.[16,17]

Concluding Remarks

Altogether, the aforementioned studies point to the complexity of the control of immune cells specific to the transient environment of the pregnant uterus. Here, we have emphasized that AM is important for establishing and maintaining a successful pregnancy. Specifically, haploinsufficiency for AM and lack of AM confer shallow trophoblast invasion into the uterine luminal epithelium during implantation and into spiral arteries, respectively. These observations have recently been recapitulated *in vitro*, whereby AM stimulates trophoblast invasion.[18] Future studies will aim to address the local control of AM dosage with the eventual goal of being able to exogenously control AM levels to promote a healthy pregnancy.

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Chapter 7: MR-proADM is a Potential Biomarker of Preeclampsia, a uNK Cell-Associated Disease[1]

Overview

Levels of the peptide hormone adrenomedullin (AM) are elevated during normal pregnancy, but whether this differs during complications of pregnancy remains unresolved. AM can be quantified by measuring its preprohormone byproduct, midregional pro-adrenomedullin (MR-proADM). MR-proADM has shown prognostic value as a biomarker of heart failure, sepsis, and community-acquired pneumonia. Given the relevance of AM to pregnancy, we tested the hypothesis that MR-proADM provides a biomarker for preeclampsia. We find that MR-proADM plasma concentrations are blunted in severe preeclampsia and that MR-proADM is similarly effective as established biomarkers endoglin and placental growth factor at discriminating patients with severe preeclampsia from controls.

Introduction

Preeclampsia is a common pregnancy complication characterized by hypertension and proteinuria.[1] Identification of novel serum biomarkers for this potentially dangerous condition is highly desirable. The peptide hormone adrenomedullin (AM) has garnered attention as a candidate biomarker of preeclampsia because its levels are elevated during

[1]Reprinted with permission from: Matson BC, Corty RW, Karpinich NO, Murtha AP, Valdar W, Grotegut CA & Caron KM. Midregional pro-adrenomedullin plasma concentrations are blunted in severe preeclampsia. *Placenta*. 2014;35:780-783.

pregnancy[2] but often altered in pregnancy complications.[3] Additionally, polymorphisms in the *ADM* gene have been associated with preeclampsia.[4]

Data from AM loss- and gain-of-function mouse models support a role for AM in placentation. Remodeling of uterine spiral arteries, conduits of the uteroplacental circulation, is critical for proper placentation[5] and is accomplished in part by crosstalk between uterine natural killer (uNK) cells and trophoblast cells of the placenta.[6] Compared to wild type placentas, $Adm^{-/-}$ placentas exhibit fewer uNK cells and insufficient spiral artery remodeling.[7] Placentas from an animal model of AM overexpression ($Adm^{hi/hi}$)[8] concordantly exhibit more uNK cells than wild type placentas and upregulate expression of uNK cell-secreted signaling molecules.[7] $Adm^{+/-}$ intercrosses commonly produce embryos demonstrating fetal growth restriction and morphological abnormalities, both common consequences of placental disorders.[9]

These data underscore the role of AM in placentation and its potential as a biomarker of preeclampsia. Previous studies arrived at inconsistent conclusions about changes in AM levels in preeclampsia,[3] likely due to the brief half-life of AM[10] and its interaction with a binding protein.[11] Recently, an assay was developed to detect midregional pro-adrenomedullin (MR-proADM), a cleaved byproduct of the preprohormone that is in direct correlation with AM.[12] Here, we measured MR-proADM plasma concentrations in women with severe preeclampsia and compared this precursor peptide to previously established biomarkers of preeclampsia.

Materials and Methods

Study design and patient samples

The study was approved by the Institutional Review Boards of Duke University and the University of North Carolina at Chapel Hill. Thirty patients with preeclampsia and 30 patients without preeclampsia delivering at Duke University Hospital with available plasma

samples were selected from the Pregnancy Outcomes: Blood and Tissue Repository study, which collected biological specimens from patients at the time of presentation and delivery. We subsequently limited our experimental group to women with severe preeclampsia by excluding women with mild preeclampsia (n=5). Additionally, women providing postpartum plasma samples (n=12), women carrying twin pregnancies (n=3), and smokers (n=7) were excluded from the original cohort. Former smokers were considered nonsmokers.

Immunoassays

Endoglin and placental growth factor (PlGF) plasma concentrations were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN) (Table 7-S1). Plasma samples were diluted 1:5 for endoglin and 1:2 for PlGF. MR-proADM plasma concentrations were measured undiluted by Phadia Immunology Reference Laboratory (Portage, MI) using a commercial immunofluorescent assay (B·R·A·H·M·S KRYPTOR, Hennigsdorf, Germany) (Table 7-S1).

Statistics

Student's t-tests were performed in GraphPad Prism 5.0. F tests were performed in R. Logistic regression and ROC analysis were performed using R package glm with no cross validation. P values < 0.05 were considered statistically significant.

Results

Table 7-1 summarizes the clinical characteristics of the patients whose plasma was analyzed in this study (Figure 7-1A). Plasma samples from controls and from women with severe preeclampsia were analyzed by ELISA for conventional biomarkers of preeclampsia. As expected,[13] soluble endoglin plasma concentrations were elevated in women with severe preeclampsia (Figure 7-1B), and placental growth factor (PlGF) plasma concentrations were blunted in women with severe preeclampsia (Figure 7-1C). After confirming that plasma from women with severe preeclampsia adhered to conventional biomarker paradigms, plasma

samples were then analyzed by a sandwich immunoassay for MR-proADM. In women with severe preeclampsia, MR-proADM concentrations were blunted (Figure 7-1D). While plasma freezer storage time differed significantly between groups, storage time did not correlate with MR-proADM concentration (r^2=0.004, data not shown).

To compare endoglin, PlGF, and MR-proADM as biomarkers of severe preeclampsia, we generated logistic regression models for each of these biomarkers alone and in combination. We then analyzed these models by generating receiver operating characteristic (ROC) curves and comparing their respective areas under the curve (AUC) (Figure 7-2). Based on AUCs, we concluded that, in this dataset, endoglin (AUC=0.73) and MR-proADM (AUC=0.69) classified patients similarly, while PlGF is slightly more informative (AUC=0.85). In multiple logistic regression, endoglin and MR-proADM together are better discriminators than either alone (AUC=0.80), as are PlGF and MR-proADM together (AUC=0.87).

Discussion

As predicted by the *Adm*-deficient mouse model,[7,9,14] we found blunted MR-proADM plasma concentrations in women with severe preeclampsia. Interestingly, during our revision process, Wellmann et al. reported elevated MR-proADM in preeclampsia using a similarly sized cohort.[15] However, their study did not distinguish between mild and severe preeclampsia and included two key groups of patients that we excluded from our study: patients carrying multiple pregnancies and smokers. Both twins and smoking have been shown to upregulate *Adm* expression, which could explain the finding of elevated AM plasma concentrations in their patients.[16-18]

Moreover, we found that MR-proADM is similarly effective as endoglin and PlGF at discriminating between patients with severe preeclampsia and controls. However, as acknowledged as a methodology limitation in both studies, the disease prevalence is higher

in the study population than in the general population. Additionally, both studies collected plasma samples late in pregnancy, while an ideal biomarker would detect preeclampsia early in pregnancy before the clinical disease becomes apparent. Ultimately, the data presented here warrant further investigation of MR-proADM at earlier gestational ages with the ultimate goal of predicting which women will develop preeclampsia later in pregnancy.

TABLES

	Control (n=18)	Severe PE (n=15)	*p* val.[b]
Age (years)	33.8 ± 4.5 (25-41)	28.5 ± 6.6 (21-41)	0.01
BMI at delivery	31.4 ± 6.7 (23.2-48.6) (n=17)	38.2 ± 9.2 (25.6-54.0)	0.03
Gravidity	3 (3-4)	2 (1-3)	0.02
Parity	1 (1-2)	0 (0-2)	0.14
Ethnicity			
White	12	7	
Black	3	8	0.99
Asian	3	0	0.07
Gestational age at delivery (weeks)	38.7 ± 0.8 (37.2-39.7)	33.9 ± 4.2 (27.4-39.9)	<0.001
Labor status			
Not in labor	14	10	
In labor	4	5	0.48
Mode of delivery			
Cesarean	16	12	
Vaginal	2	3	0.48
Blood pressure			
Systolic (mm Hg)	115.9 ± 13.1 (110-148)	164.7 ± 13.8 (143-186)	<0.001
Diastolic (mm Hg)	72.5 ± 9.1 (57-92)	103.9 ± 8.4 (90-115)	<0.001
Proteinuria			
Protein dip	0.28 ± 0.75 (0-3)	2.6 ± 1.1 (1-4) (n=10)	<0.001
Past medical history			
Hypertension	0	7	
Pregnancy-related morbidity			
Gestational diabetes	1	4	0.12
HELLP syndrome	0	3	
Chorioamnionitis	0	1	
Birthweight (grams)	3488 ± 393 (2980-4710)	2309 ± 1191 (590-4850)	<0.001
Fetal growth restriction	0	3	
Freezer storage time (days)	426.1 ± 295.8 (133-1024)	837.2 ± 322.0 (155-1099)	<0.001

Data are presented as mean ± standard deviation (range). Gravidity and parity are presented as median (interquartile range). The n is provided if different from the group n due to unavailable data. BMI, body mass index; HELLP, hemolysis, elevated liver enzymes, low platelets.
[a]Standard diagnostic criteria for severe preeclampsia were used: a systolic blood pressure ≥160 mm Hg and/or a diastolic blood pressure ≥110 mm Hg and proteinuria >500 mg/24 hours.
[b]Student's t-test for continuous variables, F test for categorical variables.

Table 7-1. Clinical characteristics of study participants.

	Control	PE	*p* val.[a]
Endoglin (ng/mL)	16.5 ± 9.6 (2.2-32.0) (n=17)	25.3 ± 13.1 (4.2-46.4) (n=12)	0.046
PlGF (pg/mL)	317.3 ± 211.7 (15.1-784.4)	113.4 ± 120.1 (5.9-406.4) (n=11)	0.007
MR-proADM (nmol/L)	1.6 ± 0.6 (0.8-2.9)	1.2 ± 0.5 (0.3-2.0)	0.041

Data are presented as mean ± standard deviation (range). Plasma samples that were too concentrated or too dilute to be detected within the range of the ELISA standard curve for endoglin and PlGF, respectively, were excluded from analysis. Grubbs' test detected one significant outlier in the PlGF concentrations in the PE group; this outlier was also excluded from analysis.
[a]Student's t-test.

Table 7-2. Plasma concentrations of endoglin, PlGF, and MR-proADM in controls and women with severe preeclampsia.

FIGURES

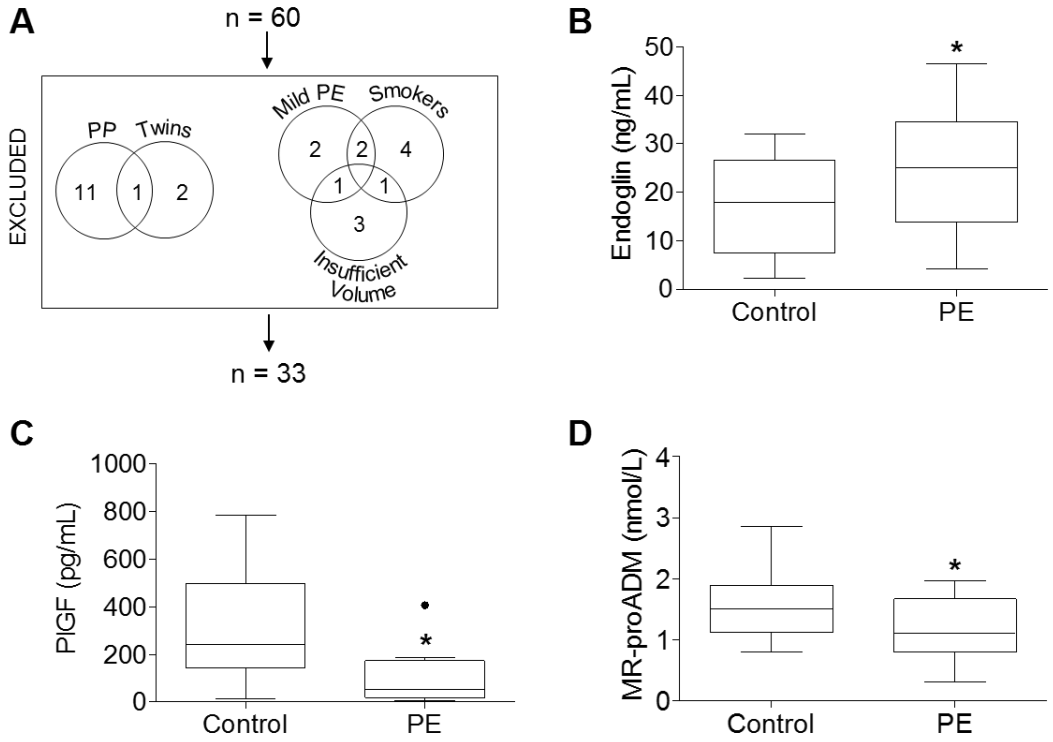

Figure 7-1. Biomarkers of preeclampsia. (A) Patients excluded from the original 60 patient cohort and (B-D) plasma concentrations of (B) endoglin, (C) placental growth factor (PlGF), and (D) MR-proADM measured by ELISA (A, B) or sandwich immunoassay (C) in controls and women with severe preeclampsia (PE). Boxes extend from the 25th to the 75th percentile. Whiskers extend 1.5 times the interquartile range below and above the 25th and 75th percentiles, respectively. Horizontal lines dividing the boxes identify the median of each group. Significant outliers, determined by Grubbs' test, are identified by a solid black dot and excluded from the analysis in this figure. Four plasma samples were too concentrated to be detected within the range of the endoglin ELISA standard curve; one of these samples belonged to the control group, while the other three belonged to the PE group. Likewise, four plasma samples were too dilute to be detected within the range of the PlGF ELISA standard curve; all four of these samples belonged to the PE group. These too-concentrated or too-dilute samples were excluded from analysis. Groups were compared by Student's t-test. *p=0.046 (A) and 0.007 (B), and 0.041 (C). PP, postpartum.

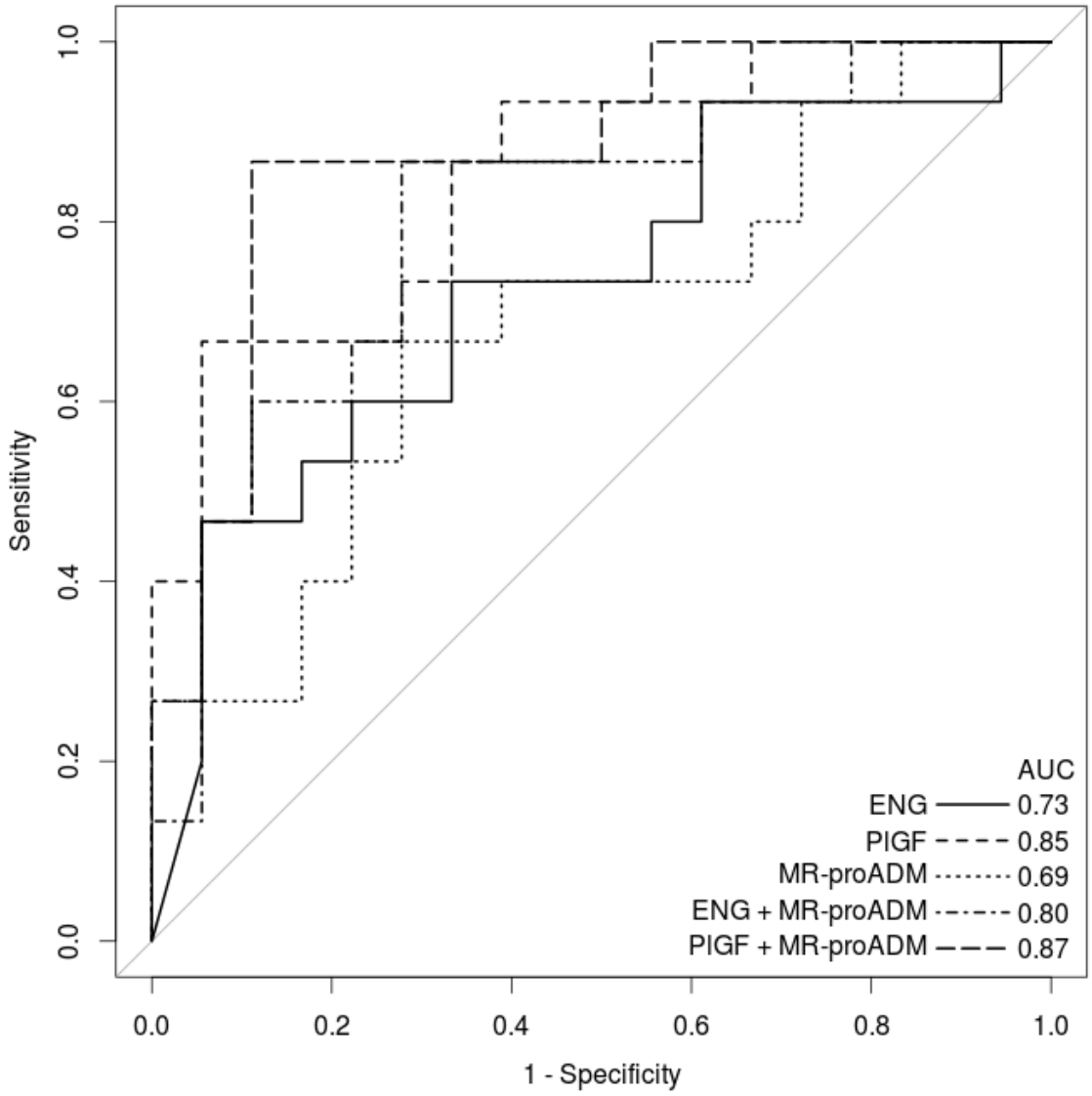

Figure 7-2. ROC curves for five logistic regression models of current severe preeclampsia based on biomarker concentrations. Plus signs separate covariates. Samples that fell above (endoglin, n=4) or below (PlGF, n=5) the ELISA standard curve were equated with the upper and lower bounds, respectively, of the standard curve. ENG, endoglin.

Supplementary Table

Endoglin (R&D Systems, DNDG00)	
detection limit	0.007 ng/mL
range	0.007 ng/mL – 10 ng/mL
specificity	No interference of the following recombinant proteins: human activin A, activin RIA, activin RIIA, activin RIIB, BMPR-1A, BMPR-1B, BMP-2, BMP-4, BMP-5, BMP-6, follistatin 288, follistatin 300, follistatin 315, inhibin A, inhibin B, LAP, TGF-α, TGF-β1, TGF-β1.2, TGF-β2, TGF-β3, TGF-β RII, TGF-β RIII; mouse BMPR-1A, BMPR-1B; rat agrin; porcine TGF-β2; amphibian TBF-β5. No interference of the following natural proteins: human TGF-β1; porcine TGF-β1.
intra-assay coefficient of variation (CV)	2.8 – 3.2% (3 samples; n=20 replicates each)
inter-assay coefficient of variation (CV)	6.3 – 6.7% (3 samples; n=40 assays each)
recovery	average 101%
linearity (EDTA plasma, 1:4 dilution)	average 107% of expected
PlGF (R&D Systems, Catalog Number DPG00)	
detection limit	< 7 pg/mL
range	< 7 pg/mL – 1000 pg/mL
specificity	No interference of the following recombinant proteins: human ANG, AR, CNTF, β-ECGF, EGF, Epo, FGF acidic, FGF basic, FGF-4, FGF-5, FGF-6, FGF-7, G-CSF, GM-CSF, sgp130, GROα, GROβ, GROγ, HB-EGF, HGF, IFN-γ, IGF-I, IGF-II, IL-1α, IL-1β, IL-1 ra, IL-1 sRI, IL-1 sRII, IL-2, IL-2 sRα, IL-3, IL-3 sRα, IL-4, IL-4 sR, IL-5, IL-5 sRβ, IL-6, IL-6 sR, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, LAP, LIP, M-CSF, MCP-1, MIP-1α, MIP-1β, β-NGF, OSM, PD-ECGF, PDGF-AA, PDGF-AB, PDGF-BB, PTN, RANTES, SCF, SLPI, TGF-α, TGF-β1, TGF-β3, TGF-β sRII, TNF-α, TNF-β, sTNF RI, sTNF RII, VEGF; mouse GM-CSF, IL-1β, IL-3, IL-4, IL-5, IL-5 sRα, IL-6, IL-7 IL-9, IL-10, IL-13, LIF, MIP-1α, MIP-1β, SCF, TNF-α; amphibian TGF-β5; chicken TGF-β3.

	No interference of the following natural proteins: bovine FGF acidic, FGF basic; human PDGF, TGF-β1; porcine PDGF, TGF-β1, TGF-β2. Recombinant human PlGF/VEGF heterodimer cross-reacts 5%. Recombinant human PlGF-2 cross-reacts 50%. Recombinant human VEGF R1/Flt-1/Fc Chimera interferes at concentrations greater than 2000 pg/mL.	
intra-assay coefficient of variation (CV)	2.8 – 3.2% (3 samples; n=20 replicates each)	
inter-assay coefficient of variation (CV)	6.3 – 6.7% (3 samples; n=40 assays each)	
recovery	average 101%	
linearity (EDTA plasma, 1:4 dilution)	average 107% of expected	
MR-proADM (BRAHMS Kryptor MR-proADM Assay via Phadia Immunology Reference Laboratory)		
detection limit	0.05 nmol/L	
range	0.05 nmol/L – 10 nmol/L	
sensitivity	0.25 nmol/L	
specificity	No interference of: cTNI up to 400 ng/mL; myoglobin up to 852 ng/mL; CKMB up to 204 ng/mL; PCT up to 50.2 ng/mL; MR-proANP up to 905 pmol/L; CT-proET1 up to 409 pmol/L; CRP up to 110 µg/mL.	
intra-assay coefficient of variation (CV)	Concentration	CV
	0.2 nmol/L – 0.5 nmol/L	≤10%
	0.5 nmol/L – 2 nmol/L	<4%
	2 nmol/L – 6 nmol/L	<2%
	> 6 nmol/L	<3.5%
inter-assay coefficient of variation (CV)	Concentration	CV
	0.2 nmol/L – 0.5 nmol/L	≤20%
	0.5 nmol/L – 2 nmol/L	≤11%
	2 nmol/L – 6 nmol/L	≤10%
	> 6 nmol/L	≤6%

Table 7-S1. Laboratory immunoassay specifications.

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Chapter 8: Conclusions and Future Directions

Summary of Results

In **Chapter 7**, we built upon the Caron laboratory's past investigations into AM and the maintenance of pregnancy in mice by expanding our studies into clinical samples from human patients. Specifically, we analyzed changes in AM in women with preeclampsia, a hypertensive disease of pregnancy characterized by dysregulation of uNK cells and insufficient spiral artery remodeling in the placenta. We performed a case-control study using plasma samples from the Pregnancy Outcomes: Blood and Tissue Repository study at Duke University, initially including 30 pregnant women with preeclampsia and 30 normotensive pregnant women. We then limited our study to women with singleton pregnancies with or without severe preeclampsia who did not smoke and who donated plasma prior to delivery.

First, we confirmed that two of the best-available biomarkers for preeclampsia, soluble endoglin (sEng) and placental growth factor (PlGF), were significantly changed in the expected directions in our patients with severe preeclampsia. We then analyzed these clinical samples for mid-regional pro-adrenomedullin (MR-proADM), finding that MR-proADM concentrations were lower in women with severe preeclampsia – a conclusion consistent with the Caron lab's AM-deficient placenta mouse model, which demonstrates preeclampsia-like phenotypes. Finally, we generated receiver operating characteristic (ROC) curves for each individual biomarker tested and then in combination with each other. We found that MR-proADM was similarly effective at discriminating between cases and controls

as sEng and PlGF and that adding information about MR-proADM to both sEng and PlGF in isolation improved the ability of each of these biomarkers to distinguish between cases and controls.

Future Directions

In the late 1990s and early 2000s, several groups assessed changes in AM in preeclampsia using several different assays, including ELISAs and radioimmunoassays.[1] However, these groups were unable to come to a consensus on the directionality of change in preeclampsia. In the mid-2000s, an assay was developed to detect MR-proADM, a stable, proteolytically cleaved byproduct of pre-pro-AM peptide, providing hope for more consistent, reliable measurement of AM in human plasma.[2] Given the preeclampsia-like phenotype of *Adm* null placentas,[3] we were curious whether MR-proADM levels were consistently lower in women with preeclampsia.

To our knowledge, we are one of only three groups to analyze changes in MR-proADM in preeclampsia. While we found blunted levels of MR-proADM in preeclampsia, the other two published studies found higher levels of MR-proADM in preeclampsia. The first, from Switzerland, examined a similarly sized cohort (n=27) but excluded women with a diagnosis of preeclampsia prior to admission and included twin pregnancies.[4] The second, from Norway, studied a larger cohort (n=105) but did not exclude twin pregnancies.[5] Neither of the two European studies collected data on the cigarette smoking status of study participants. In our study, we purposefully excluded women with multifetal gestations and women who smoked, because twin and triplet pregnancies have higher levels of AM than singleton pregnancies, and cigarette smoke upregulates AM.[6-8]

In all three studies, gestational age at delivery was significantly different between cases and controls. Controlling for gestational age at collection and at delivery is difficult with small cohort sizes as well as in the absence of established reference intervals, which

were unavailable at the time of these studies. However, trimester-specific MR-proADM intervals in healthy pregnancy were published this year.[9] As expected, Joosen et al. found that MR-proADM increases steadily throughout pregnancy and then drops precipitously in the postpartum period. While these trimester-specific intervals are helpful, gestational week-specific intervals would be better and would facilitate a more informed interpretation of these three studies.

It is helpful to start by assessing changes in MR-proADM during the active disease state, but ultimately, preeclampsia is a clinical diagnosis. Therefore, the goal of these biomarker studies is to identify a biomarker that can predict the development of preeclampsia early in pregnancy to identify patients who require higher levels of surveillance. To this end, we plan to perform a secondary analysis of the National Institute of Child Health and Human Development's Maternal-Fetal Medicine Units Network Combined Antioxidant and Preeclampsia Prediction (CAPPS) trial, which concluded that vitamin C and E supplementation beginning in the late first or early second trimester did not affect preeclampsia rates.[10] We have access to first, early second, late second, and third trimester plasma samples from 626 women in this trial and plan to measure MR-proADM longitudinally across pregnancy to determine if there are differences between healthy pregnancies and those that develop preeclampsia. It is our hope that we may be able to identify MR-proADM as a biomarker that can triage patients *early* in pregnancy, as sEng, soluble Flt1, and PlGF perform well late in pregnancy but cannot predict cases and controls early enough to merit clinical use.

Concluding Remarks

In **Part II**, we have highlighted the importance of uNK cell-trophoblast cell interactions in placentation, paying particular attention to the effects of AM on the uNK cell population and spiral artery remodeling and implicating AM in the pathophysiology of

preeclampsia. We have also drawn parallels between the Caron laboratory's previously published *Adm*-deficient placenta mouse model and MR-proADM status in women with preeclampsia. Further studies on changes in MR-proADM during early pregnancy are necessary to determine whether MR-proADM is truly predictive of preeclampsia and can be used in the clinical setting.

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