Elevated Peritoneal Fluid TNF- α Incites Ovarian Early Growth Response Factor I Expression and Downstream Protease Mediators: A Correlation With Ovulatory Dysfunction in Endometriosis

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Reproductive Sciences
20(5) 514-523
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DOI: 10.1177/1933719113477479
rs.sagepub.com

Abstract

Endometriosis-associated infertility manifests itself via multiple, poorly understood mechanisms. Our goal was to characterize signaling pathways, between peritoneal endometriotic lesions and the ovary, leading to failed ovulation. Genome-wide microarray analysis comparing ovarian tissue from an in vivo endometriosis model in the rat (Endo) with controls (Sham) identified 22 differentially expressed genes, including transiently expressed early growth response factor I (*EgrI*). The *EgrI* regulates gene requisites for ovulation. The *EgrI* promoter is responsive to tumor necrosis factor- α (TNF- α) signaling. We hypothesized that altered expression of ovarian EGR1 is induced by elevated peritoneal fluid TNF- α which is upregulated by the presence of peritoneal endometriosis. Endo rats, compared to controls, had more peritoneal fluid TNF- α and quantitative, spatial differences in *EgrI* mRNA and EGR1 protein localization in follicular compartments. Interactions between elevated peritoneal fluid TNF- α and over-expression of follicular *EgrI*/EGR1 expression may affect downstream protease pathways impeding ovulation in endometriosis. Preliminary studies identified similar patterns of EGR1 protein localization in human ovaries from women with endometriosis and compared to those without endometriosis.

Keywords

early growth response factor I, *EgrI* gene/ EGR1 protein, endometriosis, ovary, ovulation, tumor necrosis factor- α

Introduction

Growth of endometrial tissue in ectopic locations outside of the uterine lining is the hallmark of a gynecological disease called endometriosis, affecting 10% to 15% of women of reproductive age.¹ The presence of endometriotic lesions in the peritoneal cavity is commonly associated with pain and subfertility, yet mechanisms causing these conditions in endometriosis are diverse and not well characterized.¹⁻⁶

Pituitary-ovarian dysfunction manifested as abnormal patterns of luteinizing hormone (LH) secretion including a delayed and reduced amplitude LH surge and LH receptor (LHR) disorder contribute to infertility in women with endometriosis.^{3,7-11} Reduced circulating estradiol concentrations during the preovulatory phase, reduced estradiol and progesterone during the early luteal phase and anomalous estrogen receptor, progesterone receptor and progesterone resistance have also been described.^{3,12} Such anomalies, directly or indirectly, may account for impaired follicular growth, reduced dominant follicle size, fewer mature ovarian follicles, and decreased ovulation in women with endometriosis.^{6,9,12,13}

The process of ovulation is normally controlled through multiple temporal inputs, including protein and steroid hormones, metabolic signals, intrafollicular paracrine factors from the theca, mural, and cumulus granulosa cells and the oocyte.¹⁴ Animal models replicating signs and symptoms of human disease have provided opportunities for in-depth characterization of mechanisms involved in the process of ovulation in women with endometriosis.¹⁵

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Initiating with a peak in serum estradiol from the ovary, causing the pituitary LH surge and culminating in follicular rupture and extrusion of an oocyte, rat models facilitate studies of mechanisms of human disease. Such studies include intracellular signaling, gene regulation, and tissue remodeling in the ovary.¹⁶ The LH surge induces transient expression of numerous ovarian genes, including early growth response factor 1 (*Egr1*) which is requisite for ovulation. Subsequently, LH-induced *Egr1* transcription in the ovary activates expression of other genes involved in ovulation.^{17,18} Then *Egr1* promotes expression of LHR as well as temporal differences in protease and inhibitor expression and activity in the ovarian follicle, which are important for ovulation.¹⁹

In addition, the *Egr1* promoter is highly responsive to diverse biochemical signals including inflammatory cytokines such as tumor necrosis factor- α (TNF- α).^{20,21} Elevated levels of TNF- α in the peritoneal fluid of women with endometriosis compared to those without the condition may induce *Egr1* gene expression as seen in several common inflammatory diseases.^{20,22,23}

We therefore hypothesized that abnormal signaling in the pituitary–ovarian pathway and/or inflammatory factors in the peritoneal environment from endometriotic lesions alter regulation of ovarian EGR1 protein and downstream mediators requisite for ovulation, which correlate with ovulatory dysfunction in endometriosis. The goal of this study was to examine serum LH levels, ovarian LHRs, and the concentration of peritoneal fluid TNF- α in relation to ovarian *Egr1* expression and ovulatory dysfunction in an established model of endometriosis. Further to initiate translation of these data to the human, cell-specific localization of EGR1 in ovaries from women with and without endometriosis was evaluated.

Materials and Methods

Endometriosis Model Surgeries

Mature female Sprague-Dawley rats (250 g; Harlan, Madison, Wisconsin) were acclimated to the vivarium for 14 days prior to surgery. The peritoneal endometriosis (Endo, $n = 12$) and control (Sham, $n = 12$) surgeries were performed as previously described and routinely used in our laboratory.^{24–26} Briefly, in Endo rats, the left uterine horn and ovary were removed and four 2-mm sections (implants) of the uterine horn were autotransplanted into every other arterial cascade of the small intestine beginning at the cecum. Sham rats also had the left uterine horn and ovary removed without transplantation of uterine sections. One week after surgery, daily evaluation of vaginal cytology confirmed the rats had normal 4- to 5-day reproductive cycles in order to facilitate growth of the endometriotic implants. The use of animals in these experiments was approved by the University of Missouri Institutional Animal Care and Use Committee and in accord with the National Research Council's Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press).

Four weeks from the date of surgery following growth and establishment of the peritoneal implants, experiments began. A luteinizing hormone receptor hormone (LHRH) agonist (40 μ g, Sigma-Aldrich, St Louis, Missouri) was used to synchronize, not superovulate, the rats reproductive cycles as routinely performed in our laboratory.^{25–26} Between 8:00 and 10:00 AM on the morning of the first estrus after synchronization (~ 6 –8 hours postovulation) as evidenced by the presence of cornified epithelial cells in the vaginal lavage, rats were euthanized by CO₂ asphyxiation.

Sample Collection and Processing From the Model

Blood was collected by aortic puncture and aspiration (Sardstedt Monovette Syringe, Newton, North Carolina). Serum was isolated by centrifugation at 13 000 g for 30 minutes and stored at -80°C for analysis of serum LH concentrations. Serum LH was quantified in triplicate by the University of Virginia Center for Research in Reproduction, Ligand Assay and Analysis Core Facility National Institutes of Child Health and Human Development (NIH, SCCPRR Grant U54-HD28934, Charlottesville, Virginia) using a sandwich immunoradiometric assay.

Peritoneal fluid was collected by injecting 1 mL of sterile phosphate-buffered saline, pH 7.4, into the peritoneal cavity, massaging the abdomen and aspirating as much fluid as possible. After centrifugation to remove cell debris, the clear, straw-colored fluids were frozen at -80°C for subsequent quantification of TNF- α . The TNF- α was quantified in peritoneal fluid aspirates in triplicate using an enzyme-linked immunosorbent assay kit specific for rat TNF- α (RayBioTech, Norcross, Georgia). The concentration of TNF- α in peritoneal fluid was determined by the standard curve method and normalization against total protein concentration in each sample.

The remaining ovary was excised and surrounding adnexa was removed. One-half of each ovary was immediately flash frozen in liquid nitrogen for whole genome expression analysis. The remaining half of the ovary was formalin fixed, routinely processed, and paraffin embedded for spatial, cell-specific messenger RNA (mRNA), and protein localization. The presence of oocytes in ampulla of the oviducts of all rats further documented the postovulatory stage of the reproductive cycle.

Genome-Wide Microarray Analysis of Ovarian Gene Expression

Total RNA was isolated from approximately ~ 25 mg of rat ovarian tissue (approximately one-quarter of the frozen ovary) using the Qiagen RNeasy Mini Kit (Valencia, California) as per the manufacturer's instructions, including the optional on-column DNase digestion with RNase-free DNase (Qiagen). RNA was eluted from the column with 40 μ L of RNase-free water. This elution was repeated using the first eluate to increase the RNA concentration and yield. Quality and quantity of the total RNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Total RNA was amplified and biotinylated using the NuGen wt-ovation Pico RNA Amplification Kit (San Carlos, California). Biotinylated complementary RNA was hybridized to the Illumina RatRef-12 Expression Bead Chip (San Diego, California). This genome-wide array contains 22,523 probes selected from the National Center for Biotechnology Information Reference Sequence database. An Illumina BeadArray Reader identified differential gene expression between Endo and Sham rat ovaries, which Asuragen (Austin, Texas) quantified.

Microarray Results Validated Using Quantitative Real-Time Polymerase Chain Reaction

To validate the microarray results and quantify the selected genes, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. Total RNA was isolated from another quarter of the same frozen rat ovary (~25 mg) with the same protocol used to isolate RNA for the microarray. The final total RNA volume was 35 μ L. RNA quality was analyzed by an Agilent Bioanalyzer at the University of Missouri–Columbia DNA Core. The ovarian RNA (500 ng) was reverse transcribed into complementary DNA (cDNA) using the Roche First strand cDNA synthesis kit for qRT-PCR (Basel, Switzerland). The resulting cDNA was diluted 10-fold for qRT-PCR.

The qRT-PCR was performed in a 25 μ L reaction volume that contained 1 μ L of diluted cDNA, 1 μ L primer mix and 12.5 μ L Sybr green buffer from SuperArray Bioscience Inc (Bethesda, Maryland) and assayed in an ABI 7500 Smart Cycler (Applied Biosystems, Carlsbad, California). Gene expression of 18 s ribosomal RNA facilitated normalization of the target gene expression. Primers for *Egr1* and 18 s were purchased from SuperArray Bioscience Inc.

Cell-Specific Localization of *Egr1* mRNA in the Ovary

Spatial localization of *Egr1* mRNA in the ovary was identified by fluorescent in situ hybridization (ISH). All reagents and equipment used were ISH dedicated and free from RNAases. Formalin-fixed, processed, paraffin-embedded, ovarian tissues were sectioned at 8 μ m specifically for ISH. Tissues on Super Frost slides (Fisher Scientific) were deparaffinized and rehydrated through graded series of xylene, ethanol, and water washes. A pretreatment buffer of 10 mmol/L Tris (pH 7.6), 1 mmol/L EDTA, and 0.05% Tween at 95°C for 20 minutes permeabilized the tissues for hybridization. Endogenous peroxide was blocked using 0.3% H₂O₂ in methanol for 20 minutes.

Biotinylated oligonucleotide probes were designed using PrimerQuest software (Integrated DNA Technologies, Coralville, Iowa) for use as sense (GTA ATT TCA GTC GTA GTG ACC ACC T) and antisense (AGG TGG TCA CTA CGA CTG AAA TTA C) DNA probes with 100% identity to rat *Egr1* in exon 2 (NM_012551.2). Tissues were hybridized overnight at 37°C with a hybridization buffer containing 20% 20 $\times$ sodium citrate buffer (SSC), 10 mg/mL bovine serum albumin, 20% w/v dextran sulfate, 10 mg/mL sonicated salmon sperm, and the oligonucleotide probe at 1.0 ng/mL.

The next day, tissues were washed in 1:50 saline–SSC wash at 45°C for 20 minutes then rinsed in Tween 20 Tris-Buffered Saline. Tissue sections were blocked against nonspecific avidin binding at 37°C for 30 minutes with 1 $\times$ ISH blocking solution (Vector Laboratories, Burlingame, California). Tissue sections were then incubated for 30 minutes with fluorescent avidin tagged with fluorescein isothiocyanate (Vector Laboratories), counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (Invitrogen, Eugene, Oregon), and cover slipped with anti-fade mounting medium (Vector Laboratories).

Hybridization staining was quantified using a conventional fluorescent microscope (Zeiss Axiophot, 114 Zeiss Axiopath Systems, Carl Zeiss MicroImaging, Inc, Thornwood, New York). Immunofluorescent localization in the ovarian follicular theca and granulosa cells was quantified morphometrically by area fraction as described previously.²⁵ Data are presented as mean intensity per area $\pm$ standard error of the mean (SEM) as arbitrary fluorescence units.

Cell-Specific Localization of *EGR1* Protein in the Ovary

Spatial localization of the *EGR1* protein was determined by fluorescent immunohistochemistry (IHC). A rabbit anti-*EGR1* monoclonal antibody (1:1000; 0.035 μ g/mL; Cell Signaling Technology, Danvers, Massachusetts, cat no. 4153), biotinylated secondary antibody (Vector Laboratories, cat no. BA-1000), fluorescent avidin (FITC, Vector Laboratories, cat no. A-2011), and 4',6-diamidino-2-phenylindole as routinely performed by our laboratory.^{25,26} Immunolocalization of *EGR1* protein was quantified by morphometric analysis as described for ISH and used in our laboratory. Mean intensity of fluorescent staining per area $\pm$ SEM are reported in arbitrary fluorescence units.²⁵

Cell-Specific Localization of Luteinizing Hormone Receptors

Spatial localization of LHRs was determined by IHC using a rabbit monoclonal antibody (1:200; 1 μ g/mL) Santa Cruz Biotechnology Inc (Santa Cruz, California, cat no. sc-25828). Localization of the LHR was also measured and quantified as described. Again, data are reported as mean intensity per area $\pm$ SEM as arbitrary fluorescence units.²⁵

Human Ovarian Tissue

Our Health Sciences Institutional Review Board for Human Subjects approved use of human ovarian tissue sections from women with endometriosis (n = 5) and women without endometriosis (n = 8) from formalin-fixed, paraffin-embedded archival tissues. The diagnosis of endometriosis was confirmed surgically and the study pathologist (SRF), using hematoxylin and eosin stained tissue sections, confirmed ovarian morphology and the presence of endometriosis. Tissue sections containing antral follicles were selected for immunohistochemical staining for detection of *EGR1* protein as described for *EGR1* protein localization in the rat ovary.

Table 1. Differentially Expressed Genes in Ovaries from Endometriosis (Endo) Compared to Control (Sham) Rats.^a

Symbol	Description	Fold Change (Endo vs Sham)	P Value
Atf3	Activating transcription factor 3	2.3	.0036
Klf4	Kruppel-like factor 4 (gut)	2.3	.0011
Cry1	Cryptochrome 1 (photolyase-like)	2.1	.008
Egr1	Early growth response 1	2.1	.0039
OSP94_predicted	Osmotic stress protein 94 kDa	2.1	.0026
Dusp1	Dual specificity phosphatase 1	1.7	.0024
Slc23a2	Vitamin C transporter	1.5	.0044
LOC500298	Similar to ANUBL1 protein	1.5	.0075
Galnt12_predicted	N-acetylgalactosaminyltransferase 12	1.5	.0069
Klf7_predicted	Krüppel-like factor 7	1.5	.0072
Jun	Proto-oncogene (c-jun)	1.5	.0025
Prcc_predicted	Papillary renal cell carcinoma	1.5	.0081
Pdha1	Pyruvate dehydrogenase complex E1- α polypeptide	1.5	.0081
LOC300967	Transmembrane protein 108 (Tmem180)	-1.5	.0036
Vamp5	Vesicle-associated membrane protein 5	-1.6	.0041
LOC366689	similar to ribosomal protein L21	-1.6	.008
LOC501605	similar to 40 S ribosomal protein S2	-1.6	.0003
Ddt	D-dopachrome tautomerase	-1.6	.0003
Gsta4_predicted	Glutathione S-transferase, alpha 4 (predicted)	-1.6	.0089
LOC317486	Similar to RIKEN cDNA 1700045119	-1.7	.0099
LOC293679	Withdrawn by Rat Genome database	-1.5	.0044
LOC499812	Withdrawn by Rat Genome database	-1.6	.0063

^a The Illumina RatRef-12 genome wide expression Bead Array Chip (San Diego, California) contains 22 523 probes of which 22 genes were differentially expressed in Endo and Sham ovaries with a *P* value $\leq .001$ and ≥ 1.5 -fold change in expression.

Statistical Analyses

Statistical analyses to determine differences in expression detected by the microarray were performed by Asuragen Inc.²⁷ Genes considered differentially expressed required a *P* value $< .001$ and a 1.5-fold or greater difference.

One 96-well plate was sufficient to analyze all ovarian RNA by qRT-PCR. Relative mRNA expression for *Egr1* was calculated using the $2^{-\Delta\Delta Ct}$ method²⁸ with rat pituitary copy DNA as the endogenous control tissue.

Data from the analyses of serum LH levels, peritoneal fluid TNF- α concentrations, and morphometric analyses of *Egr1* mRNA, EGR1 protein, and LHR localization were normally distributed, and therefore differences in these factors between Endo and Sham rats were analyzed using Student *t* test and reported as the mean $\pm$ SE. Correlation between TNF- α and EGR1 within Endo and within Sham was determined using polynomial regression. Data are shown as mean fluorescent intensity per area in arbitrary units. A *P* value of $< .05$ was considered significant.

Results

Genome-Wide Microarray and qRT-PCR Analysis of Ovarian Gene Expression

Of the 22 523 genes tested on the microarray, 22 genes were differentially expressed when considering a stringent *P* value $\leq .001$ and a greater than 1.5-fold change in expression (Table 1). A total of 13 gene transcripts were upregulated, while 9 gene

transcripts were downregulated in Endo versus Sham rat ovaries. Two genes differentially expressed in endometriosis were removed from further consideration as records of these genes had been withdrawn from the rat genome database.

Of the differentially regulated genes, *Egr1* was chosen for further study (Endo/Sham microarray fold change = 2.1, *P* = .0039; Table 1) due to its relevance in ovulation. As measured by qRT-PCR, relative levels of *Egr1* mRNA in Endo rat ovaries (1.78 ± 0.08) were 1.25-fold higher than Sham rats (0.53 ± 0.08 ; *P* = .0024).

Cell-Specific Localization of *Egr1* mRNA And Protein

More *Egr1* mRNA localized in the theca cells of antral follicles from Endo rats (22.10 ± 1.90) versus Shams (11.36 ± 0.88 ; *P* = .001) and correspondingly, more EGR1 protein localized in Endo rats (17.67 ± 3.06) versus Shams (12.52 ± 1.66 ; *P* = .032) rats as determined by ISH/IHC, respectively, and morphometric analyses (Figures 1A and 2). In the granulosa cells of antral follicles, more *Egr1* mRNA localized in Endo rats (18.32 ± 1.35) than Shams (11.24 ± 0.56 ; *P* = .002), yet EGR1 protein localization was similar in Endo (15.19 ± 1.00) compared to Sham (13.12 ± 1.52 ; *P* = .28; Figure 1B).

Cell-Specific Localization of EGR1 Protein in Human Ovaries

The patterns of EGR1 protein localization in the granulosa and thecal cells of antral follicles from human ovaries from women with and without endometriosis (Figure 3) were similar to those

Figure 1. The early growth response factor 1 (*Egr1*) mRNA and EGR1 protein quantification in thecal and granulosa cells of Endo versus Sham rat ovaries as measured by quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA). A, More *Egr1* mRNA and EGR1 protein localized in the thecal cells of Endo rat ovaries. B, More *Egr1* mRNA but not EGR1 protein localized in the granulosa cells of Endo rat ovaries.

quantified in the endometriosis model in the rat. Follicles from women with endometriosis had more EGR1 protein in the thecal cell layer compared to women without endometriosis while similar levels of EGR1 protein were found in the granulosa cells from all ovaries tested.

The thecal cells of ovaries from women with endometriosis (100%) stained 7-fold more brightly for EGR1 protein than those of controls, where 7-fold less staining was observed in thecal cells from some women (38%) without endometriosis. However, these data need to be characterized further with a larger sample size.

Serum LH Concentrations and Cell-Specific Localization of LHRs

There was no difference in the serum concentrations of LH at the time of euthanasia (~6-8 hours postovulation) between

Endo and Sham rats. Serum concentrations of LH in Endo rats were 2.30 ± 1.38 ng/mL and in Sham rats were 2.02 ± 1.35 ng/mL ($P = .139$).

Endo and Sham LHR localization in the thecal and the granulosa cell compartments of antral follicles was similar as determined by IHC and morphometric analysis. Antral follicle thecal cell LHR in Endo rats was 25.90 ± 2.86 and similar to Sham rats 25.18 ± 1.98 ($P = .895$). Likewise antral follicle granulosa cell LHR in Endo rats (23.51 ± 1.51) was similar to that found in Sham rats (23.29 ± 5.20 ; $P = .964$).

Peritoneal Fluid TNF- α

Rats with endometriosis had significantly more TNF- α (164.36 ± 10.28 pg/mL) in their peritoneal fluid than Shams (116.25 ± 11.68 pg/mL; $P = .008$). The correlation coefficient (relationship between 2 properties where a coefficient of -1 indicates strong negative correlation, while $+1$ suggests strong positive correlation) comparing peritoneal fluid TNF- α with ovarian *Egr1* in Endo rats was 0.880 with an $r^2 = .774$ (the proportion of the variance in TNF- α attributable to the variance in *Egr1*). Yet comparing peritoneal fluid TNF- α with ovarian *Egr1* in Sham rats there was an inverse correlation, with a correlation coefficient of -0.601 and an $r^2 = .361$ (Figure 4).

Discussion

A Role for EGR1 in Ovulatory Dysfunction Leading to Infertility in Endometriosis

Molecular pathways set into motion when a preovulatory follicle receives an ovulatory LH stimulus to initiate a complex, interrelated cascade of events leading to ovulation.¹⁴ We hypothesized that in endometriosis, abnormal pituitary-ovarian pathways and/or the inflammatory peritoneal environment function to regulate expression of ovarian *Egr1*/EGR1 and downstream mediators requisite for ovulation and correlate with ovulatory dysfunction.

The EGR1 is a phosphoprotein with an extended transcriptional activation domain on the N-terminus and a DNA-binding domain, consisting of 3 zinc finger motifs. These motifs bind to the GC-rich sequence 5'-GCGGGGCG-3' and are rapidly activated in many cell types by numerous stimuli.²⁹ The EGR1, via coupling with extracellular signals, is believed to elicit long-term responses by altering expression of EGR1 target genes. The targeted gene products are then responsible for the physiological or pathophysiological alterations that result from cellular stimulation and EGR1 biosynthesis.²⁹ In a tissue-/cell-specific manner and conserved across species from rabbit to human, EGR1 reportedly participates in diverse roles regulating the cell cycle, growth and signaling, tissue remodeling, differentiation, and apoptosis.^{30,31}

The *Egr1* in the ovary binds promoter elements of many of genes altering pathways important for ovarian function. Knocking out *Egr1* in the mouse demonstrated that EGR1 is

Figure 2. Localization of early growth response factor I (*Egr1*) mRNA and EGR1 protein by in situ hybridization (ISH; A-F) and immunohistochemistry (IHC; G-L) in Endo and Sham rat ovaries. More *Egr1* mRNA and EGR1 protein localized in the thecal cells of Endo (A, G) versus Sham (B, J) rat ovaries. White arrows indicate the ovarian thecal cell layer. Panels B, E, H, I show sense negative controls. Panels C, G, I, L show 4',6-diamidino-2-phenylindole stain for nuclei.

essential for ovulation.³²⁻³⁴ Further, disruption of the normally transient expression of *Egr1* and subsequent misregulation of the highly regulated, requisite expression of proteases and their

inhibitors may impede ovulation. For example, abnormal expression of cathepsin L in ovarian granulosa cells and/or alternations in a disintegrin and metalloproteinase with

Figure 3. Immunolocalization of early growth response factor I (EGR1) protein by immunohistochemistry (IHC) in ovaries from women with and without endometriosis. More EGR1 protein localized in ovarian thecal cells from women with endometriosis than those without endometriosis. (TA = tunica albuginea, T = thecal cells [green stain= EGR1], BM = basement membrane, GC = granulosa cells, L = lumen of follicle).

thrombospondin motifs 1 and tissue inhibitor of matrix metalloproteinases 1 (TIMP1) in granulosa cells and thecal cells have been associated with ovarian dysfunction.³⁴⁻³⁶ As demonstrated in our laboratory, TIMP1 synthesized and secreted from endometriotic lesions localizes in ovarian thecal cells of Endo but not Sham rats.²⁵ Endometriotic TIMP1 localization in the theca is associated with decreased follicular growth, decreased ovulation, and decreased fecundity.^{25,26,37} The EGR1 may further disrupt the protease/inhibitor milieu necessary for the tissue remodeling required for ovulation and exacerbate this problem.

Serum LH and Ovarian LHR

In women with endometriosis, infertility might be a consequence of a defect in mechanisms mediating serum LH and ovarian LHR action.^{7,11} In women, serum LH levels are lower, the LH surge delayed and ovarian LHR concentrations remain low across the menstrual cycle compared to controls.^{7,9,10} Endometriosis models using the rat have elucidated mechanisms whereby experimental alterations in the normal LH surge or LHRs block the expression of *Egr1* and its effects on

downstream expression of proteases and their inhibitors necessary for ovulation.¹⁷ These models have shown that increasing serum LH concentrations cause induction of *Egr1* expression in ovarian thecal and granulosa cells in the rat.¹⁸ In turn, ovarian *Egr1* expression regulates expression of the ovarian LHR gene.¹⁹

Yet in our study, the similarity of serum LH concentrations between Endo and Sham rats suggests that overexpression of *Egr1* in the ovary is not induced via a serum LH surge mechanism. In turn, elevated ovarian EGR1 concentrations in endometriosis did not promote expression of LHR as per the typical role of EGR1.¹⁹ Plausible interpretations include lack of an LH-EGR1-LHR mechanism in ovarian dysfunction in endometriosis or the use of LHRH to synchronize the estrous cycles in this model. Also, other studies did not report the reproductive cycle stage at the time of sample collection. All of the rats in our study were euthanatized between 8:00 and 10:00 AM on the morning of the estrus stage. Hence, the different hormonal milieu of each reproductive stage may be related to the varying results. Different testing methods or different rat strains may also account for these discrepancies. These disparities remain to be resolved.

Figure 4. Correlation of ovarian early growth response factor 1 (EGR1) and levels of tumor necrosis factor- α (TNF- α) in peritoneal fluid in Endo compared to Sham rats. The correlation coefficient comparing peritoneal fluid TNF- α with ovarian *Egr1*/EGR1 gene and protein in Endo rats was 0.880, $R^2 = .774$ (diamonds). The correlation coefficient comparing peritoneal fluid TNF- α with ovarian *Egr1*/EGR1 mRNA and protein in Sham rats was $-.606880$, $R^2 = .361$ (circles). This indicates a phenomenon in endometriosis whereby when peritoneal fluid TNF- α increases, ovarian EGR1 expression escalates potentially altering extracellular matrix remodeling requisite for ovulation, a phenomenon not found in Sham rats.

Peritoneal Fluid TNF- α

Women with endometriosis have elevated levels of TNF- α in their peritoneal fluid, which may be necessary for establishment and maintenance of endometriosis. Peritoneal fluid TNF- α has also been associated with infertility through inhibition of sperm motility and function and oocyte development.^{38,39} As in women with endometriosis, our studies have shown that rats used to model endometriosis-associated subfertility have significantly more TNF- α in their peritoneal fluid compared to controls. Others have also reported elevated levels of peritoneal fluid TNF- α in Endo rats correlate with increased numbers of activated macrophages.⁴⁰ TNF- α signaling through receptor binding increases the level of *Egr1* in cultures of primary human fibroblasts.¹⁹ Hence TNF- α may bind to and increase expression of *Egr1* in this endometriosis model.

Thereby we propose a model whereby elevated TNF- α in peritoneal fluid that bathes the ovary incites EGR1 biosynthesis. Then, abnormal amounts and localization of EGR1 in the ovary disrupt normal cell signaling and gene expression required for ovulation. In turn, excessive ovarian thecal cell EGR1 may mediate tissue remodeling in the ovary in endometriosis essential for ovulation via inference with the metalloproteinase/inhibitor pathway^{16,25} as well as other downstream pathways regulating growth, proliferation, differentiation, and/or apoptosis. Yet at this time, these observations represent only an association, although a provocative one, between *Egr1* and ovulatory dysfunction in endometriosis. Ongoing studies in

our laboratory are addressing the challenge to establish cause-effect relationships relevant to the outcomes.

EGR1 in Human Ovaries

Intriguingly, the patterns of EGR1 protein localization in the granulosa and thecal cells of antral follicles from human ovaries from women with and without endometriosis paralleled those quantified in the endometriosis model in the rat. While these preliminary data needs to be further characterized with a larger sample size, this is the first report of increased amounts of EGR1 protein in human ovarian thecal cells during the periovulatory period in women with endometriosis compared to controls.

Conclusions

Endometriosis is a common disease among reproductive age women and elucidating the mechanism behind reduced fecundity seen in these women is important. The etiology of infertility in endometriosis likely involves numerous mechanisms that may be confounded by interpretation. Abnormal hormone production, receptor function, peritoneal fluid inflammatory mediators and ovulatory dysfunction may individually or synergistically play a role in endometriosis-associated infertility.^{8,9,12,13,38,41-43}

Collectively, results from this study support a correlation between elevated concentrations of TNF- α in peritoneal fluid, upregulation of follicular EGR1, and its association with altered expression of downstream genes and proteins involved in ovulatory dysfunction in endometriosis, directly in the endometriosis model and indirectly in women. Further insights into the outcomes of elevated TNF- α in peritoneal fluid and increased EGR1 in the ovary in endometriosis and its effect on ovarian function may help develop novel therapeutic targets for ovulatory dysfunction via the use of inhibitors or antagonists to TNF- α action.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Support for this work was provided by the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD Grant 1R01HD057445 (to KST).

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