

UCSF

UC San Francisco Previously Published Works

Title

Changes in Eutopic Endometrial Gene Expression During the Progression of Experimental Endometriosis in the Baboon, Papio Anubis¹

Permalink

<https://escholarship.org/uc/item/822762zb>

Journal

Biology of Reproduction, 88(2)

ISSN

0006-3363

Authors

Afshar, Yalda
Hastings, Julie
Roqueiro, Damian
[et al.](#)

Publication Date

2013-02-01

DOI

10.1095/biolreprod.112.104497

Peer reviewed

Changes in Eutopic Endometrial Gene Expression During the Progression of Experimental Endometriosis in the Baboon, *Papio Anubis*¹

Yalda Afshar,^{3,4} Julie Hastings,^{3,5} Damian Roqueiro,⁶ Jae-Wook Jeong,⁷ Linda C. Giudice,⁸ and Asgerally T. Fazleabas^{2,7}

⁴Department of Obstetrics and Gynecology, Cedars-Sinai Medical Center, Los Angeles, California

⁵Human Reproductive Sciences Unit, Medical Research Council, Queen's Medical Research Institute, Edinburgh, United Kingdom

⁶Department of Bioengineering, University of Illinois, Chicago, Illinois

⁷Department of Obstetrics, Gynecology and Reproductive Biology, College of Human Medicine, Michigan State University, Grand Rapids, Michigan

⁸Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, San Francisco, California

ABSTRACT

Endometriosis is associated with aberrant gene expression in the eutopic endometrium of women with disease. To determine if the development of endometriotic lesions directly impacts eutopic endometrial gene expression, we sequentially analyzed the eutopic endometrium across the time course of disease progression in a baboon model of induced disease. Endometriosis was induced in baboons ($n = 4$) by intraperitoneal inoculation of autologous menstrual endometrium. Eutopic endometria were collected during the midsecretory phase (Days 9–11 postovulation) at 1, 3, 6–7, 10–12, and 15–16 mo after disease induction and compared with tissue from disease-free baboons. RNA was hybridized to Human Genome U133 Plus 2.0 Arrays, and data were extracted using Gene-Chip Operating Software. Subsequently, both Gene Set Enrichment Analysis and Ingenuity Pathways Analysis were used to find biological states that have a statistically significant enrichment concomitant with pairwise comparison of human endometriosis arrays. Within 1 mo of induction of the disease, 4331 genes were differentially expressed ($P < 0.05$). Hierarchical clustering revealed self-segregation into two groups—a) 1, 3, and 10–12 mo and b) 6–7 and 15–16 mo—together with controls. Clustering analysis at each stage of disease validated dysregulation of several signaling pathways, including Nodal-like receptor, EGF, ERK/MAPK, and PI3/AKT. Sequential analysis of the same animals during disease progression demonstrated an early disease insult and a transitory dominance of an estrogenic phenotype; however, as the disease progressed, a progesterone-resistant phenotype became evident. Furthermore, we demonstrate a 38.6% differential gene expression overlap with endometrial samples in the midsec-

tory phase from women with endometriosis, concomitant with similar dysregulation in human disease candidate genes *Fos*, *Nodal*, *Suclg2*, and *Kras*, among others. Molecular changes in the eutopic endometrium, associated with endometriosis, are directly impacted by endometriotic lesions, providing strong evidence that it is the disease rather than inherent defective endometrium that results in aberrant gene expression in the eutopic endometrium. Furthermore, this baboon model provides a powerful means whereby the early events associated with the pathology of disease and the resulting infertility may be elucidated.

baboon, endometriosis, endometrium, primate, transcriptome

INTRODUCTION

Endometriosis is a major cause of infertility and an often-painful disease characterized by the presence of endometrial tissue outside the uterine cavity [1, 2]. The disease affects one in 10 women of reproductive age [3], 50%–60% of women and teenage girls with pelvic pain [4], and up to 50% of women with infertility [3].

Multiple factors have been implicated in endometriosis-associated infertility, including significant alterations in the molecular markers of endometrial receptivity in women [5] and baboons [6, 7]. The endometrial transcriptome, cellular signaling, and biochemical pathway modulation differ in the endometrium from women with endometriosis from those of disease-free women [5, 8]. Comparison of the eutopic endometrial transcriptome of women with moderate and severe disease and those without disease reveals differences in all phases of the menstrual cycle but primarily in the proliferative, early secretory, and midsecretory phases, with a persistence of the proliferative phenotype and enhanced cell survival in disease [5]. The attenuated progesterone response in the midsecretory phase of women and baboons with endometriosis is reflected in the altered expression of progesterone-regulated genes [5, 6, 9]. Using our baboon model, we propose that following the establishment of endometriosis, the eutopic endometrium undergoes an early coordinated series of aberrant changes during the window of uterine receptivity compared to disease-free controls, similar to those observed in human endometrium in the setting of existing endometriosis.

To date, a vast numbers of eutopic gene arrays have been investigated in women with a history of established disease at clinical presentation. These women carry a heavy disease burden and/or a disease load that is poorly defined due to

¹Supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development/National Institute of Health cooperative agreement (U54 HD 40093 to A.T.F. and U54 HD55764 to L.C.G.) as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.

²Correspondence: Asgerally T. Fazleabas, Center for Women's Health & Reproduction, Michigan State University, College of Human Medicine, 333 Bostwick Ave. NE, Room 4027 Grand Rapids, MI 49503-2532. E-mail: asgi@hc.msu.edu

³These authors contributed equally to this work.

Received: 12 September 2012.

First decision: 4 October 2012.

Accepted: 19 December 2012.

© 2013 by the Society for the Study of Reproduction, Inc.

eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

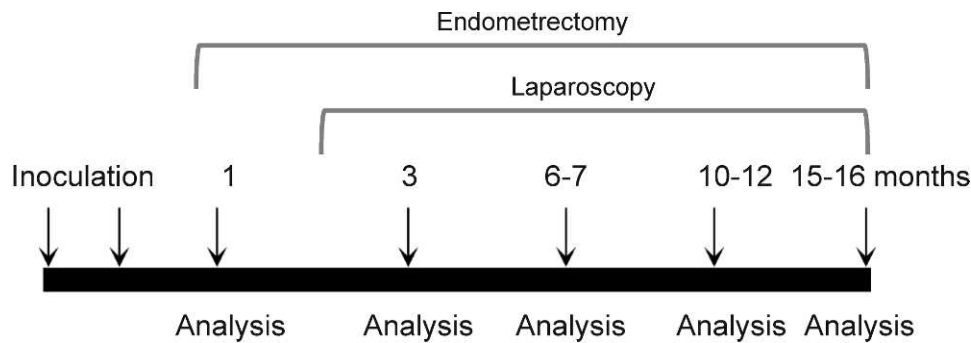


FIG. 1. Endometriosis was experimentally induced in female *Papio anubis* (baboons) with documented regular menstrual cycles by intraperitoneal inoculation with menstrual endometrium. Endometrium was harvested on Days 1–2 of menses immediately prior to laparoscopy. At the subsequent menses, the animals underwent a second laparoscopy and endometrial reseeded at the same ectopic site, the uterine fundus, the cul de sac, the ovaries, and the pouch of Douglas. Following each laparoscopy, a laparotomy was performed, and eutopic endometrial tissue was harvested. The progression of disease was monitored in each animal by consecutive laparoscopies at 1 (n = 2), 3 (n = 4), 6 (n = 4), 9–12 (n = 4), and 15–16 (n = 4) mo after inoculation, during the window of uterine receptivity (Days 9–11 postovulation [PO] in the baboon). At 15–16 mo following the second inoculation, the animals were euthanized, and a necropsy was carried out to obtain all of the associated reproductive tissues within the peritoneal cavity.

extensive time to diagnosis. As such, it is not possible to clearly demonstrate that the aberrant gene expression that has been reported is a product or consequence of endometriosis.

We and others have developed the baboon (*Papio anubis*) as a model for the study of endometriosis [10–12]. Our model allows a tightly controlled environment of experimentally induced endometriosis in baboons with documented regular menstrual cycles. Intraperitoneal inoculation with autologous menstrual endometrium results in the formation of endometriotic lesions in the baboons with histological and morphological characteristics similar to those in women [13]. Individual differences in progression and symptoms of the disease create difficulties in conducting objective long-term studies in women. Furthermore, endometriosis only occurs naturally in humans and nonhuman primates.

Previously, we performed a small-scale microarray to determine if immunological aberrations were evident in the eutopic endometrium of baboons 6 mo following experimental induction of endometriosis [14]. In the current study, we have performed extensive microarray analysis using the Affymetrix Gene Arrays to examine changes in eutopic endometrial gene expression during the development of experimental endometriosis in baboons from 1 to 15–16 mo after disease; the expression pattern was then compared to that in disease-free model and subsequently in baboons with spontaneous endometriosis.

MATERIALS AND METHODS

Animals and Induction of Experimental Endometriosis

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois, Chicago, and Michigan State University. Endometriosis was experimentally induced in four female *P. anubis* baboons by intraperitoneal inoculation with menstrual endometrium on two consecutive menstrual cycles, as previously described [7]. Animals were nulliparous with documented regular menstrual cycles and had not undergone any previous surgeries. Two baboons with spontaneous endometriosis were included in this study with an unknown duration of disease. Menstrual endometrium was harvested on Days 1–2 of menses using a Unimar Pipelle (Cooper Surgical Inc., Shelton, CT) immediately prior to laparoscopy. Under laparoscopic guidance, approximately 1 g of menstrual tissue and fluid was deposited from the Pipelle at four sites: the pouch of Douglas, the uterine fundus, the cul de sac, and the ovaries. At the subsequent menses, the animals underwent a second laparoscopy and endometrial reseeded at the same ectopic sites. The progression of disease was monitored in each animal by consecutive laparoscopies and video recording at 1 (n = 2), 3 (n = 4),

6–7 (n = 4), 10–12, (n = 4), and 15–16 (n = 3) mo after inoculation during the window of uterine receptivity (Days 9–11 postovulation [PO] in the baboon [Fig. 1]). After laparoscopic entry, a complete systemic survey of the abdomen and pelvic cavity was performed, and the number, color, and position of each visible lesion were digitally documented [15]. The presence of peritoneal fluid, extent of adhesions, level of surface vasculature, scar tissue, and corpora lutea were noted. Following each laparoscopy, a laparotomy was performed, and eutopic endometrial tissue was harvested [13]. Unlike in women where endometrial biopsies yield sufficient tissue for analyses, in baboons we obtain only a very limited amount of tissue with pipelle biopsies, primarily due to the size of the uterus. Therefore, in order to utilize a valuable animal model to its fullest extent, we obtain endometrial tissue following laparotomy using a procedure referred to as endometriectomy, which yields the most amount of tissue to maximize our analyses [12]. At 15–16 mo following the second inoculation, the animals were euthanized as required by the IACUC approval, which permits a maximum of four invasive surgeries, and a necropsy was carried out to obtain all of the associated reproductive tissues within the peritoneal cavity.

Collection and Processing of Tissue

Blood samples were collected daily from Days 7 through 16 postmenstruation of menstrual cycles, during which surgery was performed. Serum estradiol (E2) was measured by radioimmunoassay (DSLabs, Webster, TX). The serum E2 peak was taken as Day –1 of ovulation, and the day of ovulation was designated as Day +1 [16]. Eutopic endometrial tissues were harvested between Days 9 and 11 PO and were snap frozen in liquid nitrogen for RNA extraction. Eutopic endometrium from the functionalis layer was consecutively harvested by endometriectomy from the same animals following experimental induction of endometriosis (Fig. 1).

Disease-Free Control Animals

Control endometrium was similarly harvested from animals (n = 4) with no previous surgeries and with no visible disease between Days 9 and 11 PO. Disease-free (DF) control animals were subjected to laparotomies. Laparoscopy was done prior to the laparotomy to confirm the complete absence of spontaneous endometriosis in the control animals [15].

Microarray

Eutopic endometria were homogenized in TRI-ZOL reagent (Invitrogen, Carlsbad, CA), and RNA was extracted. Total RNA was then subjected to DNase digestion to remove genomic DNA and was purified using the RNeasy Kit (Qiagen, Valencia, CA). RNA purity was confirmed by 260/280-nm absorbance ratios and analysis on an Agilent Bioanalyzer, following which cDNA was prepared according to the Affymetrix microarray preparation protocol (Affymetrix, Santa Clara, CA). Individual samples were hybridized to Affymetrix Human Genome U133 Plus 2.0 Arrays and scanned using an HR3000 scanner and data were extracted using the Affymetrix Gene-Chip Operating System V1.1.

Microarray Analysis

The gene expression data were normalized and background corrected with the Robust Multichip Average method [17] using R/Bioconductor [18]. Nonspecific filtering was performed by obtaining the overall variability of each probe set across arrays. Probe sets with low variability between different time points were safely discarded since they do not add value in the inference of differential expression of their target genes [19]. To find differentially expressed probe sets, the limma package in R/Bioconductor was utilized [20]. Limma uses linear models to analyze a designed experiment and to determine differential expression [21]. To determine the linear coefficients of the model, a design matrix was created that included a coefficient for the difference of samples between time points, and a moderated *t*-statistic was used. The *P* value for each probe set was then corrected with the Benjamini-Hochberg algorithm for multiple hypothesis testing [22]. This method controls the expected false discovery rate and probe sets with an adjusted *P* value of <0.05 were considered differentially expressed.

Probe sets were mapped to unique Entrez Gene IDs using a custom Chip Description File (CDF) [23]. The R package “HGU133A_Hs_ENTREZG” version 13.0 (July 2010) was used in this analysis, as it provided the most current mapping between probe sets and gene Entrez IDs for the Affymetrix Human Genome U133 Plus 2.0 microarray.

Fold change, as the sole criterion for differential expression, does not provide a probabilistic estimation of the significance of the variations in gene expression. The normalization of the expression data, the use of a moderated *t*-test, and the posterior correction for multiple hypothesis testing are robust methods to obtain a list of differentially expressed genes based on the significance level of our choice (0.05). Differential expression analysis was conducted in samples between consecutive time points, such as 1 mo versus control, 3–6 mo versus 1 mo, and so forth, including the analysis of gene expression in animals with spontaneous disease versus all other time points.

Subsequently, both Ingenuity Pathways Analysis (IPA) and pathway and GO term enrichment with DAVID Bioinformatics [24] were input with the lists of differentially expressed genes between time points. These tools allowed us to identify any statistically significant enrichment of GO terms or molecular pathways between the two biological states represented by samples of different time points. Principal components analysis (PCA) and hierarchical clustering were applied to the normalized expression values of all genes in all samples. A dendrogram was added to the heat maps to illustrate the output of the hierarchical clustering. The dendrogram marks the inter-cluster distance between the different clusters. Samples whose genes have similar expression patterns clustered together.

RESULTS

Clustering Analysis

PCA is frequently used to reduce the dimensionality of a data set by reporting the principal components that accumulate the largest variance. PCA of all samples in a microarray experiment provides insights into the expression profiles of the genes in each sample. As a result of this, samples that have similar gene expression profiles cluster closely together. In our analysis, samples clustered into two major groups (Fig. 2). When we consider the average expression values of genes at each time point, the PCA clearly separates the average expression profiles by disease time point (Fig. 2A). The X-axis accumulates 71% of the variance and provides a clearer picture of the two main clusters situated to the left and right of the chart. The Y-axis is the second principal component, wherein the DF control animals and the early and transitional stages are separated from the M10–12 and M15–16 of disease.

In a similar manner, the average expression profiles of all genes at each time point, including four samples from DF control animals, were used for an unfiltered hierarchical clustering analysis. The average profiles self-segregated into two major groups. Hierarchical clustering demonstrated similarities in eutopic endometrial transcriptome between animals at 1, 3, and 10–12 mo of disease (Fig. 2B). Pairwise comparisons between dysregulated transcripts at 1, 3, and 10–12 mo of disease revealed striking similarities. The second cluster was comprised of samples from animals at 6–7 (*n* = 4) and 15–16 (*n* = 4) mo following induction of disease, together

with those from DF control animals (*n* = 4). Animals with spontaneous disease (*n* = 2) and labeled as S1 and S2 clustered with those at 6–7 and 15–16 mo of induced disease (Fig. 2C).

The hybridization efficiency of baboon cRNA to a human array platform was investigated (Supplemental Figure S1; all Supplemental Data are available online at www.biolreprod.org). The number of detectable probes in each sample indicated a hybridization efficiency ranging from 18% to 39%, well within that expected for any human sample [25]. However, Animal 7024 (induced disease) displayed a low hybridization efficiency of less than 10% and was excluded from further analysis. Morphologically, 32 of the 48 (67%) ectopic lesions harvested contained both endometrial glands and stroma (data not shown). The disease burden was similar between animals, and we observe an average of nine lesions per animal at each laparoscopy. The majority of the lesions found 1 mo following tissue inoculation are red lesions, which change color during the disease progression. In addition, new lesions are continuously developing, and they persist up to 15 mo postinoculation [15].

Early Disease Burden

Within only 1 mo of disease induction, pairwise comparisons of eutopic endometrium demonstrated 4331 genes that were differentially expressed compared to DF animals in the eutopic endometrium. Very early after induction of disease, this large-scale dysregulation of the endometrial transcriptome was observed; 2201 genes were exclusive to the 1-mo time frame without redundancy at other time points. The largest number of members in a gene family, 17, were altered in the modulator of nonspecific immunity, nucleotide oligomerization domain-like receptors [26], within 1 mo of disease, compared to controls (Supplemental Figure S2) [27]. Between no other time points of disease, 3, 6–7, 10–12, and 15–16 mo following inoculation, was the differential gene expression as robust as at 1 mo (Fig. 3 and Table 1). A significant lack of differential regulation of the endometrial transcriptome was observed in the same animals 2 mo later or 3 mo following induction of endometriosis, whereby zero genes were differentially expressed among time points. Of note, the pairwise comparisons between DF controls and spontaneous disease demonstrated a significant gene modulation of 4032 genes.

Transitional Response

In comparisons between control and 6–7 mo of disease, only one gene, the proto-oncogene *FOS*, was differentially expressed, marking the dominance of the estrogenic phenotype. Between time points 3 to 6–7 and 6–7 to 10–12 mo of disease, 1803 and 1893 genes, respectively, were differentially expressed, *P* < 0.05; 170 genes were exclusive to the 3- to 6–7-mo transition and 266 genes to the 6–7- to 10–12-mo transition, and 92 genes overlapped between the transitions of these three time points. All other differential expression was shared with the 1-mo to control group (Fig. 3). BIOCARTA analysis demonstrated enrichment of differential expression with the five members of the costimulatory signal during T-cell activation—*Cd3d*, *Icoslg*, *Pik3R1*, *HLA-DRA*, and *CD28*—between M6–7 and M10–12 (Supplemental Table S1).

Late Response

Comparing differential gene regulation directly between the final two time points, 10–12 and 15–16 mo of disease, there are minimal alterations in the genetic signature of the endometrium. Only two genes, Interferon regulatory factor 2 (*IRF2*) and

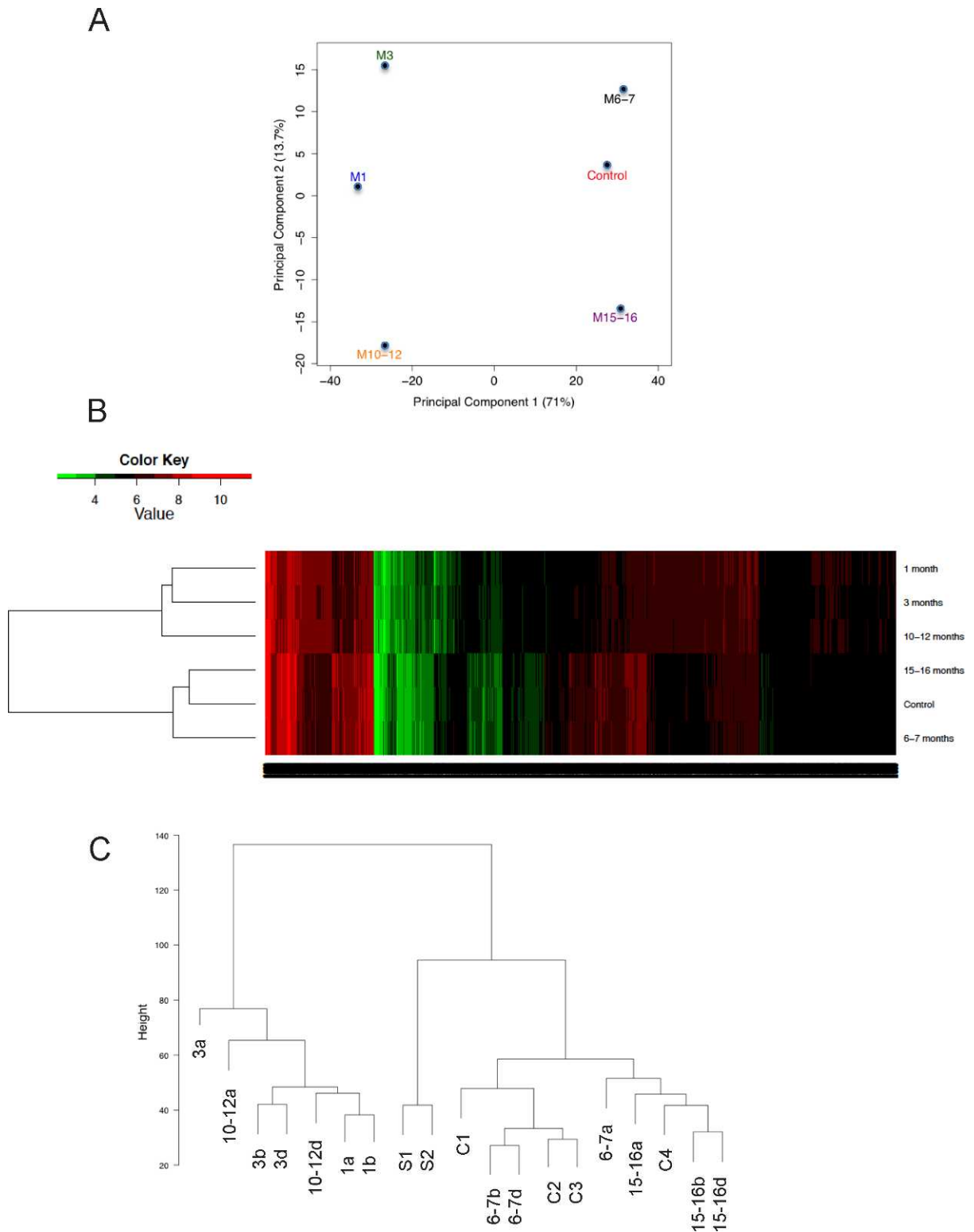


FIG. 2. **A**) PCA of the average expression levels at each time point. The first two principal components, the ones that account for the largest variance between average samples, were used to visualize the data. Samples that have similar gene expression profiles cluster close together by disease length. Using all genes on the Human Genome Affymetrix array, samples self-segregated into two major clusters. The y-axis is the second PCA component, wherein the DF controls and the early and transitional stages are separated from the M10–12 and M15–16 of disease. **B**) Unfiltered hierarchical clustering and heat mapping demonstrated similarities in eutopic endometrial transcriptome between animals at 1, 3, and 10–12 mo of disease. Pairwise comparisons between dysregulated transcripts at 1, 3, and 10–12 mo of disease revealed striking similarities. The second cluster was comprised of samples from animals 6–7 and 15–16 mo following induction of disease, together with those from DF control animals. **C**) Hierarchical clustering of each animal (sample), including baboons with spontaneous disease.

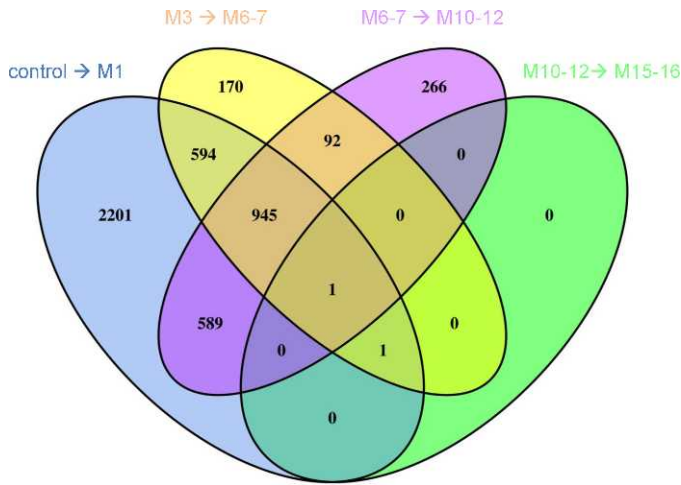


FIG. 3. The Venn diagram depicts differentially expressed genes through each transitional time point of disease and overlap between these transitions. Genes are differentially expressed with an adjusted P value <0.05 . Note that between the month (M)1–(M)3 disease points, no genes were significantly differentially expressed (Table 1).

adipoQ receptor family member III (*Paqr3*), are differentially expressed compared to DF controls, $P < 0.05$ (Supplemental Table S2). To elucidate the pattern of differential gene regulation in the eutopic endometrium of baboons over a 15–16-mo period following induction of endometriosis, no correlations were significant with disease load [15]. The level of gene dysregulation did not correlate with the number of lesions present or the type of lesion or the position of lesion present at each time point (Fig. 4).

Canonical Pathways and Networks Through Disease

Pathway analysis (KEGG and BIOCARTA) using DAVID Bioinformatics [24, 28] provided a means to quantitatively present pathways differentially modulated throughout the time course of disease. KEGG analysis demonstrated neuroactive ligand-receptor interaction (73 members, adjusted P value $<5.82E-03$), tyrosine metabolism, tight junction, cysteine and methionine metabolism, and leukocyte transendothelial migration. BIOCARTA points to PKC-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase and the role of myelin and lymphocyte protein in rho-mediated activation of serum response factor (Supplemental Table S1)

IPA evaluated the changes of the genes in disease versus control at 1, 3, 6–7, 10–12, and 15–16 mo. The degree of regulation of signaling pathways and network formations in different comparison groups depends on the total number of genes regulated in each group. Significantly regulated network functions and canonical pathways formations are demonstrated in Tables 2 and 3.

The IPA software uses computational algorithms to identify cellular networks that statistically fit the input gene list and expression values from experiments. Data sets containing the Affymetrix probe identifiers and fold changes of genes were overlaid on global molecular networks that were developed from connectivity information. Networks were then algorithmically generated, and a score was assigned. The score is used to rank networks according to how relevant they are to the genes in the input database demonstrating reproductive system development and function, cancer, and reproductive system disease as the second-highest IPA score behind cell cycle, cardiac necrosis, and cell death (Table 2). Of note, a

TABLE 1. Number of genes differentially expressed ($P < 0.05$) in aforementioned time points.

Time points compared ^a	No. of genes
Ctrl → M1	4331
M1 → M3	0
M1 → M6–7	1
M3 → M6–7	1803
M6–7 → M10–12	1893
M10–12 → M15–16	2
Ctrl vs. spontaneous	4032

^a Ctrl, control; M, months.

predominant associated network function included both reproductive system development and function (Table 3; score: 31, $P = 7.6E-04 - 1.4E-02$, 21 molecules).

Canonical pathways analysis identified the pathways that were most significant to the input data set. The significance of association was determined based on the ratio of the gene number from the data sets that maps to pathways divided by the total number of genes that map to the canonical pathways, and a P value was calculated using the Fischer exact test, which determined the probability that the association between the genes in the data set and the canonical pathway is due only to chance. Each network of pathways was set to have a maximum of 30 focus genes. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge. The intensity of the node color indicates the degree of up-regulation (red) or down-regulation (green). IPA demonstrated that during disease progression, several signaling pathways were significantly affected in a dynamic time-specific manner, namely, PI3K/AKT, EGF, and ERK signaling (Supplemental Figures S3–S5).

Pathway Analysis Reveals Altered EGF Signaling Profile in Baboons with Endometriosis

The EGF signaling pathways demonstrated key genes of interest, including *Fos*, *Map2k7*, *Jak1*, *June*, *Pik3c2a*, *Mapk3*, *Pik3cg*, *Plcg1*, *Stat1*, and *Mig6*. *Egfr*, together with *Pi3k* and *Stat3/Stat3-P*, was down-regulated following disease induction (Table 4 and Supplemental Figure S3). We observed dysregulation of several antiproliferative genes in the EGF signaling cascade. Mitogen-inducible gene 6 (*Mig6*) functions as a negative regulator of EGF mitogenic signaling, and here we demonstrate that the gene is elevated at 15–16 mo of disease and low in the early disease process, consistent with human data that noted an increase in *Mig6* in women with long-term disease [5]. Additionally, *Fos* was the single gene that was differentially regulated between DF controls and 6–7 mo of disease, consistent with our previously published data [14].

Pathway Analysis Reveals Altered PI3K/AKT Signaling Profile in Baboons with Endometriosis

Induction of disease immediately affects the PI3K/AKT signaling in baboons with endometriosis. After 1 mo of disease burden, a number of PI3K/AKT pathways genes are modulated, including *Jak1*, *Mapk3*, *Ywhaz*, *Pdpk1*, *Kras*, *Mdm2*, *Ikmke*, *Lims1*, *Jak2*, *Inpp5d*, *Bcl2*, *Pten*, *Ppp2cb*, *Gab1*, *Ppp2r3a*, *Pik3cg*, *Ppm1j*, *Ppm1l*, *Ppp2r2b*, *HLA-B*, *Rheb*, *Iga4*, and *Them4* (Table 4 and Supplemental Figure S4). Of note, the progesterone receptor-mediated signaling pathways are attenuated at 6–7 mo of disease, confirming that the

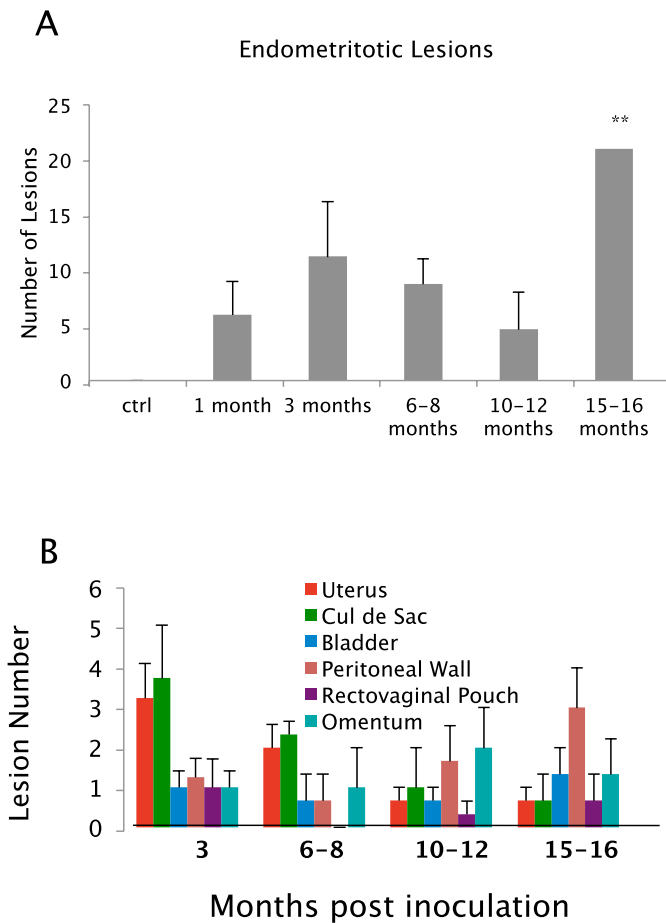


FIG. 4. After laparoscopic entry, a complete systemic survey of the abdomen and pelvic cavity was performed, and the number of each visible lesion (A) and lesion location (B) was documented. At 15–16 mo, a hysterectomy and necropsy were performed, and all lesions were documented. The total number of lesions at each time point was not significantly different until the 15–16-mo time point (** $P < 0.05$), which is the time point at which all lesions are visualized and documented in the laboratory following complete removal of all tissues.

endometrium is less responsive to progesterone as a consequence of endometriosis [29–31].

Pathway Analysis Reveals Altered ERK/MAPK Signaling Profile in Baboons with Endometriosis

Our data to date suggest that the eutopic endometrium undergoes sequential changes that are initially characterized by a dominance of E2-regulated genes early on in disease such as *FOS*. Significantly, the ERK/MAPK signaling pathways [32] are differentially expressed during disease progression. These genes include *Fos*, *Mapk3*, *H3f3b*, *Pla2g2a*, *Ppp1r3a*, *Kras*, *Ksr1*, *Ppm1j*, *Pik3cg*, *Ppm1l*, *Mknk1*, *Stat1*, *Itga4*, *Pla2g12a*, *Pik3c2a*, *Ywhaz*, *Plcg1*, *Nfatc1*, *Pla2g4c*, *Ppp2cb*, *Elf2*, *Araf*, *Ppp2r3a*, *Ppp2r2b*, *Prkg2*, and *Esr* as are described in the time course (Table 4 and Supplemental Figure S5).

Baboon Model Confirms Candidate Gene Susceptibility in Women with Endometriosis

Cross-referenced [5] pairwise hierarchical analysis with our baboon data as early as 1 mo following induction of disease confirmed significant overlap with women with endometriosis. Burney et al. [5] report 721 human genes significantly

TABLE 2. Significantly regulated network functions.

Network function	IPA score
Cell cycle, cardiac necrosis, cell death	37
Cellular assembly and organization, cellular function and maintenance	31
Cell signaling, molecular transport and mineral metabolism	31
Reproductive system development and function, cancer, reproductive system disease	31
Drug metabolism, lipid metabolism, small molecular biochemistry	28

differentially expressed in the eutopic endometrium of endometriosis compared to controls: 428 were up-regulated and 293 down-regulated at least 1.5-fold. Significantly, 278 genes, or 38.6%, overlapped in our baboon model of endometriosis. An additional 72 genes were significantly differentially expressed between human and baboon spontaneous disease (Supplemental Table S3). Intriguingly, a number of midsecretory phase candidate genes that are dysregulated in women with endometriosis [5] were also differentially expressed following disease induction in the baboon, further validating this model. These genes include *Sugcl2*, *S100a1*, *Cdkn2b*, *Cyp26a1*, *Muc3b*, *Rin3*, *Paqr3*, and *Nodal*, which were differentially regulated throughout the course of the disease. Additionally, this overlap between the baboon and human data extended to pathways detailed above (Supplemental Table S3).

DISCUSSION

The experimental induction of endometriosis in female baboons continues to establish the baboon as a viable, nonhuman primate model for endometriosis research, providing insight into the pathophysiology of a disease that affects up to 50% of women with infertility [33]. Progression of disease was monitored in our animals by consecutive laparoscopies over a period of 15–16 mo after peritoneal inoculation occurring during the window of uterine receptivity (Days 9–11 PO in the baboon). Inoculation of menstrual endometrium resulted in the formation of endometriotic lesions with both gross morphological and histological characteristics similar to those seen in women [13]. The development of these endometriotic lesions was observed in all animals that underwent intraperitoneal inoculation. Progression of disease with changes in the eutopic endometrial transcriptome as early as 1 mo after disease was associated with the establishment and maintenance of endometriotic lesions in the peritoneal environment.

Although retrograde menstruation occurs in 70%–90% of women in the reproductive age group, endometriosis is diagnosed in only 10% of this population [3, 34]. This suggests that endometriosis is a consequence of an inherent endometrial defect or a genetic predisposition. Surgical removal of endometriotic lesions as well as treatment with gonadotropin-releasing hormone (GnRH) analogs improve pregnancy rates in patients with endometriosis [35–38]. Lessey et al. [39] reported improvement in fertility observed in women with endometriosis treated with GnRH analogs and subsequent ablation of endometriotic foci. These data support the theory that an inherent endometrial dysfunction could be associated with the reduced pregnancy rates, secondary to failed implantation, in women with endometriosis.

Within only 1 m following disease induction, 4331 genes were differentially expressed in the eutopic endometrium

TABLE 3. Significantly regulated canonical pathways formations.

Physiological system development and function	P value	No. molecules
Tumor morphology	3.27E-04–9.36E-03	11
Reproductive system development and function	7.61E-04–1.45E-02	21
Behavior	8.48E-04–2.11E-02	44
Nervous system development and function	8.48E-04–2.35E-02	38
Endocrine system development and function	9.04E-04	3

compared to disease-free controls, implying a direct and acute interaction between the presence of ectopic endometriotic lesions and alterations in uterine gene signature and possibly uterine receptivity. Endometrial gene signatures, at different time points, self-segregated into clusters (Fig. 2). Differentially expressed genes in the baboon showed a high correlation with aberrant gene expression in endometrium of women with endometriosis (Supplemental Table S3).

The strength of this study lies in the ability to document the early disease process that cannot be captured in the human disease, as we have the ability to document the exact length of disease in baboons. The baboon model allows us to directly determine how the presence of ectopic lesions directly impacts eutopic gene expression as early as 1 mo after the disease and monitor the dynamic alteration of the gene signature during the progression of the disease. Our data demonstrate that the molecular changes in the eutopic endometrium associated with endometriosis directly impacts endometrial function and provides strong evidence that it is the presence of the lesions that results in aberrant a “waxing-waning” pattern of gene expression in the eutopic endometrium during disease progression. We speculate that although the lesion numbers do not change significantly during the time course of the disease (Fig. 4 [12]), the waxing-waning pattern of gene expression we observe may be associated with periods of uterine quiescence that may be conducive to pregnancy. Many functional clues can be gained from looking at individual genes and global gene expression changes in microarray data at set statistical parameters. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge with the intensity of the node color indicates the degree of up-regulation (red) or down-regulation (green) could then be followed through time (Supplemental Figures S3–S5). The EGF signaling pathway is significantly modulated in our model; members include *Fos*, *Egfr*, and *Mig6*, among others. *Fos* encodes the protein c-Fos, a member of the AP-1 family of proteins, which play a critical role in controlling cell death [14]. AP-1 can act as a transcriptional activator, or repressor [40]. *Fos* is up-regulated 10–26-fold in the eutopic endometrium of baboons with endometriosis at 3 mo and decreased by 93- and 60-fold over controls at 6 and 10–12 mo, respectively. Fifteen months after induction of endometriosis, levels of *Fos* mRNA expression were similar to those found in control tissues from animals with no disease [14]. Furthermore, *Fos* has been shown to inhibit progesterone

receptor (PR)-induced transcription [41]. We have proposed that in the very early stages of the disease, when the levels of PRA are similar in eutopic endometrium of induced animals and controls, increased levels of *Fos* may inhibit the ability of PRA to mediate the transcription of progesterone-regulated genes [42]. This interaction would provide another mechanism whereby PRA activity is inhibited; in contrast, estrogen receptor (*ESR*)-induced genes such as MMPs, *Vegf*, and *Cyr61* are expressed in the eutopic endometrium during the window of receptivity at the early stages of the disease, when we also observe the greatest change in global gene expression, even though there are no significant differences in the expression level of steroid hormone receptors during the early phase of the disease [13, 43, 44].

It has been demonstrated that the absence of *Mig6* results in the inability of progesterone to inhibit estradiol-induced uterine weight gain and estradiol-responsive target gene expression [45]. *Mig6*, also known as *Erffil* (ERBB receptor feedback inhibitor 1), regulates the duration of MAPK activation by attenuating EGFR autophosphorylation [46]. Murine genetic studies have demonstrated that this gene is a critical regulator of endometrial response to estradiol in regulating tissue homeostasis [47]. Furthermore, down-regulation of *Mig6* may be associated with conferred survival advantage of the refluxed endometrium and lead to the establishment of endometriotic lesions [48] as it becomes constitutively expressed in endometriosis and results in an increase in mitogenic signaling.

We demonstrate that the eutopic endometrium exhibits molecular abnormalities. It has been previously demonstrated the eutopic endometrium demonstrates the activation of oncogenic pathways like the rat sarcoma viral oncogene homologue (*Ras*) [2]. Activating *K-ras* mutations have also been identified in rodent models of endometriosis [49] and women with disease [31]. A screen of women with endometriosis demonstrated a polymorphism in a let-7 micro-RNA (miRNA) binding site in the 3'-UTR of *Kras* and detected a *Kras* variant allele in 31% of women with endometriosis as opposed to 5% of a large diverse control population. Inherited polymorphism of a let-7 miRNA binding site in *Kras* leads to abnormal endometrial growth and endometriosis. The LCS6 polymorphism has been described as a genetic marker for endometriosis risk [31]. Among other tumor suppressors and oncogenes, the candidate genes *Kras* and *Pten* have been implicated [50]. Of note, *Ywhaz* in the endometrium is differentially regulated in our baboon model

TABLE 4. Altered signaling profiles in baboons with endometriosis.

Canonical pathways	Molecules
PI3K/AKT signaling	JAK1, MAPK3, YWHAZ, PDPK1, KRAS, MDM2, IKKBE, LIMS1, JAK2, INPP5D, BCL2, PTEN, PPP2CB, GAB1, PPP2R3A, PIK3CG, PPM1J, PPM1L, PPP2R2B, HLA-B, RHEB, ITGA4, THEM4
ERK/MAPK signaling	MAPK3, H3F3B, PLA2G2A, PPP1R3A, KRAS, KSR1, PPM1J, PIK3CG, PPM1L, MKNK1, STAT1, ITGA4, PLA2G12A, PIK3C2A, YWHAZ, PLCG1, NFATC1, PLA2G4C, PPP2CB, FOS, ELF2, ARAF, PPP2R3A, PPP2R2B, PRKAG2, ESR1
EGF signaling	FOS, MAP2K7, JAK1, JUN, PIK3C2A, MAPK3, PIK3CG, PLCG1, STAT1

(Joshi and Fazleabas, unpublished data) and is a target of micro-RNA 451 [51, 52]. *Bcl2* and a family of pro- and antiapoptotic family members couple to the PI3K/AKT signaling cascade and provides further evidence of apoptotic resistance and enhanced survival of endometrial cells in endometriosis [53].

We have previously demonstrated high levels of *Mmp7*, *Vegf*, *Cyr61*, and *Fos* along with an altered distribution of steroid hormone receptors in both the ectopic and the eutopic endometrium of these animals [7, 42, 43, 54, 55]. The increased expression of MMPs and angiogenic factors in both the eutopic and the ectopic endometrium, coupled with the high expression of estrogen receptor (*ESR*), as demonstrated in the ERK/MAPK signaling profile, has been speculated to allow the endometrial fragments to rapidly attach and invade through peritoneal surfaces to establish lesions. Furthermore, inhibition of MMPs in rodent and primate models suppressed the development of lesions both *in vivo* and *in vitro* [56–61] and angiogenesis [62–64].

A disadvantage of our study is that we could not follow pregnancy rates in the animals because of a necropsy needing to be performed at the end of the study and the limitation of statistical power in a small group of animals. Similar to women, baboons with endometriosis have lower pregnancy rates [65, 66]. A further limitation of this study was that control tissues were collected only at a single time point and not at consecutive time points to match the time points of the disease process. There is always the possibility that consecutive laparoscopies followed by endometriectomies could alter the pattern of gene expression, but in the context of this study it is unlikely that the multiple surgeries would have influenced our results since we observe the greatest change in gene expression at 1 mo following disease induction when the animals have not had a single laparotomy.

Using a well-established nonhuman primate model, we have demonstrated that the presence of endometriotic lesions results in an altered eutopic endometrial environment very early in the disease process. By sequential analysis of the eutopic endometrium in the same animals during the progression of the disease, we have also demonstrated that early in the disease process there is a transitory dominance of an estrogenic phenotype, but as the disease progresses a more permanent progesterone-resistant phenotype results concomitant with previously established human disease patterns [5, 30].

REFERENCES

- Giudice LC. Clinical practice: endometriosis. *N Engl J Med* 2010; 362: 2389–2398.
- Bulun SE. Endometriosis. *N Engl J Med* 2009; 360:268–279.
- Eskenazi B, Warner ML. Epidemiology of endometriosis. *Obstet Gynecol Clin North Am* 1997; 24:235–258.
- Goldstein DP, deCholnoky C, Emans SJ, Leventhal JM. Laparoscopy in the diagnosis and management of pelvic pain in adolescents. *J Reprod Med* 1980; 24:251–256.
- Burney RO, Talbi S, Hamilton AE, Vo KC, Nyegaard M, Nezhat CR, Lessey BA, Giudice LC. Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology* 2007; 148:3814–3826.
- Braundmeier AG, Fazleabas AT. The non-human primate model of endometriosis: research and implications for fecundity. *Mol Hum Reprod* 2009; 15:577–586.
- Hastings J, Fazleabas A. A baboon model for endometriosis: implications for fertility. *Reprod Biol Endocrinol* 2006; 4:S7.
- Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN, Osteen K, Lessey BA, Giudice LC. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology* 2003; 144:2870–2881.
- Fazleabas AT. Progesterone resistance in a baboon model of endometriosis. *Semin Reprod Med* 2010; 28:75–80.
- D'Hooghe T, Bamba C, Suleman M, Dunselman G, Evers H, Koninckx P. Development of a model of retrograde menstruation in baboons (*Papio anubis*). *Fertil Steril* 1994; 62:635–638.
- Fazleabas AT. A baboon model for simulating pregnancy. *Methods Mol Med* 2006; 121:101–110.
- Fazleabas AT. A baboon model for inducing endometriosis. *Methods Mol Med* 2006; 121:95–99.
- Fazleabas AT, Brudney A, Gurates B, Chai D, Bulun S. A modified baboon model for endometriosis. *Ann N Y Acad Sci* 2002; 955:308–317; discussion 340–302, 396–406.
- Hastings JM, Jackson KS, Mavrogianis PA, Fazleabas AT. The estrogen early response gene FOS is altered in a baboon model of endometriosis. *Biol Reprod* 2006; 75:176–182.
- Harirchian P, Gashaw I, Lipskind ST, Braundmeier AG, Hastings JM, Olson MR, Fazleabas AT. Lesion kinetics in a non-human primate model of endometriosis. *Hum Reprod* 2012; 27:2341–2351.
- Fazleabas AT, Kim JJ, Srinivasan S, Donnelly KM, Brudney A, Jaffe RC. Implantation in the baboon: endometrial responses. *Semin Reprod Endocrinol* 1999; 17:257–265.
- Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* 2003; 31:e15.
- Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004; 5:R80.
- Hahne F, Huber W, Gentleman R, Falcon S. *Bioconductor Case Studies*. New York: Springer-Verlag; 2008.
- Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; 3:1.
- Smyth GK. Limma: linear models for microarray data. In: Gentleman RC, Irizarry R., and Huber W (eds.), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. New York: Springer; 2005:397–420.
- Benjamini Y, Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B* 1995; 57: 289–300.
- Dai M, Wang P, Boyd AD, Kostov G, Athey B, Jones EG, Bunney WE, Myers RM, Speed TP, Akil H, Watson SJ, Meng F. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. *Nucleic Acids Res* 2005; 33:e175.
- Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 2003; 4:P3.
- Wang P, Ding F, Chiang H, Thompson RC, Watson SJ, Meng F. ProbeMatchDB—a web database for finding equivalent probes across microarray platforms and species. *Bioinformatics* 2002; 18:488–489.
- Abrahams VM. The role of the Nod-like receptor family in trophoblast innate immune responses. *J Reprod Immunol* 2011; 88:112–117.
- Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M. KEGG for integration and interpretation of large-scale molecular data sets. *Nucleic Acids Res* 2012; 40:D109–D114.
- Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; 4:44–57.
- Aghajanova L, Giudice LC. Molecular evidence for differences in endometrium in severe versus mild endometriosis. *Reprod Sci* 2011; 18: 229–251.
- Aghajanova L, Velarde MC, Giudice LC. Altered gene expression profiling in endometrium: evidence for progesterone resistance. *Semin Reprod Med* 2010; 28:51–58.
- Grechukhina O, Petracco R, Popkhadze S, Massasa E, Paranjape T, Chan E, Flores I, Weidhaas JB, Taylor HS. A polymorphism in a let-7 microRNA binding site of KRAS in women with endometriosis. *EMBO Mol Med* 2012; 4:206–217.
- Velarde MC, Aghajanova L, Nezhat CR, Giudice LC. Increased mitogen-activated protein kinase/extracellularly regulated kinase activity in human endometrial stromal fibroblasts of women with endometriosis reduces 3',5'-cyclic adenosine 5'-monophosphate inhibition of cyclin D1. *Endocrinology* 2009; 150:4701–4712.
- Missmer SA, Cramer DW. The epidemiology of endometriosis. *Obstet Gynecol Clin North Am* 2003; 30:1–19, vii.
- Blumenkrantz MJ, Gallagher N, Bashore RA, Tenckhoff H. Retrograde menstruation in women undergoing chronic peritoneal dialysis. *Obstet Gynecol* 1981; 57:667–670.
- Alborzi S, Momtahan M, Parsanezhad ME, Dehbashi S, Zolghadri J,

- Alborzi S. A prospective, randomized study comparing laparoscopic ovarian cystectomy versus fenestration and coagulation in patients with endometriomas. *Fertil Steril* 2004; 82:1633–1637.
36. Littman E, Giudice L, Lathi R, Berker B, Milki A, Nezhat C. Role of laparoscopic treatment of endometriosis in patients with failed in vitro fertilization cycles. *Fertil Steril* 2005; 84:1574–1578.
 37. Sallam HN, Garcia-Velasco JA, Dias S, Arici A. Long-term pituitary down-regulation before in vitro fertilization (IVF) for women with endometriosis. *Cochrane Database Syst Rev* 2006:CD004635.
 38. Surrey ES, Schoolcraft WB. Does surgical management of endometriosis within 6 months of an in vitro fertilization-embryo transfer cycle improve outcome? *J Assist Reprod Genet* 2003; 20:365–370.
 39. Lessey BA, Young SL. Integrins and other cell adhesion molecules in endometrium and endometriosis. *Semin Reprod Endocrinol* 1997; 15: 291–299.
 40. Rouleau J, MacLeod AR, Szyf M. Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway. *J Biol Chem* 1995; 270: 1595–1601.
 41. Shemshedini L, Knauth R, Sassone-Corsi P, Pornon A, Gronemeyer H. Cell-specific inhibitory and stimulatory effects of Fos and Jun on transcription activation by nuclear receptors. *EMBO J* 1991; 10: 3839–3849.
 42. Jackson KS, Brudney A, Hastings JM, Mavrogianis PA, Kim JJ, Fazleabas AT. The altered distribution of the steroid hormone receptors and the chaperone immunophilin FKBP52 in a baboon model of endometriosis is associated with progesterone resistance during the window of uterine receptivity. *Reprod Sci* 2007; 14:137–150.
 43. Gashaw I, Hastings JM, Jackson KS, Winterhager E, Fazleabas AT. Induced endometriosis in the baboon (*Papio anubis*) increases the expression of the proangiogenic factor CYR61 (CCN1) in eutopic and ectopic endometria. *Biol Reprod* 2006; 74:1060–1066.
 44. Jackson K, Hastings JM, Brudney A, Fazleabas AT. Endometriosis alters the pattern of steroid receptor expression in the baboon endometrium. *Biol Reprod* 2004; (special issue): Abstract 8.
 45. Jeong JW, Lee HS, Lee KY, White LD, Broaddus RR, Zhang YW, Vande Woude GF, Giudice LC, Young SL, Lessey BA, Tsai SY, Lydon JP, et al. Mig-6 modulates uterine steroid hormone responsiveness and exhibits altered expression in endometrial disease. *Proc Natl Acad Sci U S A* 2009; 106:8677–8682.
 46. Ferby I, Reschke M, Kudlacek O, Knyazev P, Pante G, Amann K, Sommergruber W, Kraut N, Ullrich A, Fassler R, Klein R. Mig6 is a negative regulator of EGF receptor-mediated skin morphogenesis and tumor formation. *Nat Med* 2006; 12:568–573.
 47. Jeong JW, Lee HS, Franco HL, Broaddus RR, Taketo MM, Tsai SY, Lydon JP, DeMayo FJ. Beta-catenin mediates glandular formation and dysregulation of beta-catenin induces hyperplasia formation in the murine uterus. *Oncogene* 2009; 28:31–40.
 48. Wu Y, Strawn E, Basir Z, Wang Y, Halverson G, Jailwala P, Guo SW. Genomic alterations in ectopic and eutopic endometria of women with endometriosis. *Gynecol Obstet Invest* 2006; 62:148–159.
 49. Cheng CW, Licence D, Cook E, Luo F, Arends MJ, Smith SK, Print CG, Charnock-Jones DS. Activation of mutated K-ras in donor endometrial epithelium and stroma promotes lesion growth in an intact immunocompetent murine model of endometriosis. *J Pathol* 2011; 224:261–269.
 50. Kobayashi H, Kajiwara H, Kanayama S, Yamada Y, Furukawa N, Noguchi T, Haruta S, Yoshida S, Sakata M, Sado T, Oi H. Molecular pathogenesis of endometriosis-associated clear cell carcinoma of the ovary (review). *Oncol Rep* 2009; 22:233–240.
 51. Bergamaschi A, Katzenellenbogen BS. Tamoxifen downregulation of miR-451 increases 14-3-3zeta and promotes breast cancer cell survival and endocrine resistance. *Oncogene* 2012; 31:39–47.
 52. Vestergaard AL, Knudsen UB, Munk T, Rosbach H, Martensen PM. Transcriptional expression of type-I interferon response genes and stability of housekeeping genes in the human endometrium and endometriosis. *Mol Hum Reprod* 2011; 17:243–254.
 53. Braun DP, Ding J, Shaheen F, Willey JC, Rana N, Dmowski WP. Quantitative expression of apoptosis-regulating genes in endometrium from women with and without endometriosis. *Fertil Steril* 2007; 87: 263–268.
 54. Fazleabas AT, Kim JJ. Development. What makes an embryo stick? *Science* 2003; 299:355–356.
 55. Fazleabas AT, Strakova Z. Endometrial function: cell specific changes in the uterine environment. *Mol Cell Endocrinol* 2002; 186:143–147.
 56. Barrier BF, Bates GW, Leland MM, Leach DA, Robinson RD, Propst AM. Efficacy of anti-tumor necrosis factor therapy in the treatment of spontaneous endometriosis in baboons. *Fertil Steril* 2004; 81(suppl 1): 775–779.
 57. Bruner KL, Matrisian LM, Rodgers WH, Gorstein F, Osteen KG. Suppression of matrix metalloproteinases inhibits establishment of ectopic lesions by human endometrium in nude mice. *J Clin Invest* 1997; 99: 2851–2857.
 58. D’Hooghe T, Nugent N, Cuneo S, Chai D, Deer F, Debrock S, Kyama C, Mihalyi A, Mwenda J. Recombinant human TNFRSF1a (r-hTBP1) inhibits the development of endometriosis in baboons: a prospective randomized, placebo-and drug-controlled study. *Biol Reprod* 2006; 74: 131–136.
 59. D’Antonio M, Martelli F, Peano S, Papoian R, Borrelli F. Ability of recombinant human TNF binding protein-1 (r-hTBP-1) to inhibit the development of experimentally-induced endometriosis in rats. *J Reprod Immunol* 2000; 48:81–98.
 60. Nap AW, Dunselman GA, de Goeij AF, Evers JL, Groothuis PG. Inhibiting MMP activity prevents the development of endometriosis in the chicken chorioallantoic membrane model. *Hum Reprod* 2004; 19: 2180–2187.
 61. Sillem M, Prifti S, Koch A, Neher M, Jauckus J, Runnebaum B. Regulation of matrix metalloproteinases and their inhibitors in uterine endometrial cells of patients with and without endometriosis. *Eur J Obstet Gynecol Reprod Biol* 2001; 95:167–174.
 62. Hull ML, Charnock-Jones DS, Chan CL, Bruner-Tran KL, Osteen KG, Tom BD, Fan TP, Smith SK. Antiangiogenic agents are effective inhibitors of endometriosis. *J Clin Endocrinol Metab* 2003; 88:2889–2899.
 63. Laschke MW, Elitzsch A, Vollmar B, Vajkoczy P, Menger MD. Combined inhibition of vascular endothelial growth factor (VEGF), fibroblast growth factor and platelet-derived growth factor, but not inhibition of VEGF alone, effectively suppresses angiogenesis and vessel maturation in endometriotic lesions. *Hum Reprod* 2006; 21:262–268.
 64. Nap AW, Griffioen AW, Dunselman GA, Bouma-Ter Steege JC, Thijssen VL, Evers JL, Groothuis PG. Antiangiogenesis therapy for endometriosis. *J Clin Endocrinol Metab* 2004; 89:1089–1095.
 65. D’Hooghe TM, Bamba CS, Raeymaekers BM, Riday AM, Suleman MA, Koninckx PR. The cycle pregnancy rate is normal in baboons with stage I endometriosis but decreased in primates with stage II and stage III-IV disease. *Fertil Steril* 1996; 66:809–813.
 66. Schenken RS, Asch RH, Williams RF, Hodgen GD. Etiology of infertility in monkeys with endometriosis: measurement of peritoneal fluid prostaglandins. *Am J Obstet Gynecol* 1984; 150:349–353.