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DEVELOPMENTAL EXPOSURE OF FETAL OVARIES AND FETAL GERM CELLS TO ENDOMETRIOSIS CAUSES DIFFERENTIAL GENE EXPRESSION IN PRE-IMPLANTATION EMBRYOS OF FIRST AND SECOND GENERATION EMBRYOS OFFSPRING IN AN ENDOMETRIOSIS MODEL

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Abstract

Objective—Characterize multigenerational gene expression anomalies in 8-cell stage embryos associated with developmental exposure to endometriosis.

Design—Using an endometriosis model in rats (F0 founder generation), evaluate gene expression in F1 (fetal exposure) and F2 (fetal germ cell exposure) generation 8-cell stage embryos.

Setting—Laboratory

Animals—Endometriosis model in rats (Endo) and controls (Sham)

Interventions—F0 Endo and Sham rats were bred. Half of the pregnant rats were euthanatized on gestational day 3 (F1 8-cell stage embryos); the others gestated to term (F1 females). Adult F1 females were bred and F2 8-cell embryos collected.

Main outcome measures—Maintenance of differential gene expression in F1 and F2 generation 8-cell embryos in endometriosis.

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Results—Developmental exposure to endometriosis altered gene signaling pathways including apoptosis, cell cycle process, response to oxidative stress, negative regulation of molecular function and RNA processing. Apoptotic genes *Diablo*, *Casp3*, *Parp1*, *Cad* and *Dnaja3* were increased, *Nfkb* transcripts decreased in F1 Endo versus F1 Sham embryos. In F2 Endo versus Sham embryos, *Casp3* and *Cad* were significantly increased plus *Parp1* and *Nfkb* tended to be elevated.

Conclusions—Fetal and germ cell exposure to endometriosis alters apoptotic gene expression in first and second generation 8-cell stage embryos, supporting the hypothesis of multigenerational inheritance from exposure to endometriosis in utero.

Keywords

endometriosis; embryo; genome analysis; apoptosis; multigenerational

INTRODUCTION

Endometriosis is a benign gynecological disease found in 10% to 15% of reproductive age women in the United States (1). Ectopic growth of endometrial tissue defines this disease. The most common symptoms of endometriosis are pain and infertility. Many women with minimal, mild or moderate endometriosis experience difficulties in establishing and maintaining pregnancy [2].

Multiple reports describe familial clustering of endometriosis (2, 3). Genetic studies of 123 women with surgically documented endometriosis reported their mothers (5.9%) and sisters (8.1%) also had; but endometriosis was only found in 0.9% of controls (4). Yet this may also be interpreted as the reported rate ranged between 0.9% to 15% in the general population, and therefore, "5.9%" and "8.1%" in first-degree relatives of endometriosis patients is no different than in the general population of women. A different study of 750 women surgically documented endometriosis identified a significantly higher relative risk in sisters (5.20) and cousins (1.56) (2). It could be that the overall risk indicates polygenic and multifactorial etiologies are far more likely to be the cause than Mendelian inheritance.

A small number of putative gene deletions or mutations have been reported in women with endometriosis, which offer a genetic explanation for the familial tendency for endometriosis (2, 4, 5). Unfortunately, various methodological differences and deficiencies, including not controlling for risk factors or intermediary genetic traits, have led to publication of conflicting results further impeding our identification of a genetic basis for endometriosis (6). There has been significant difficulty in replication in different study populations suggesting ancestry may play a role in genetic mutations (5). Hence, despite the familial nature of endometriosis, no definitive multigenerational genetic link is evident.

As found in literature reviews over the past decade, many women with endometriosis have trouble in establishing and maintaining pregnancy (7, 8, 9). Mechanisms causing reduced fecundity in women with endometriosis are diverse and often multifactorial in nature (9) such that one diagnosis does not fit all. Further, these mechanisms are intricately intertwined and often confounding, thereby contributing to our lack of understanding of the effects of endometriotic lesions on fertility. Endometriosis may exert its impact on fertility in women at numerous levels of reproduction. Beginning with the hypothalamic – pituitary - ovarian axis and coursing through ovarian function, oocyte quality, embryo development and implantation, endometrial function, endometriosis may affect one or many of these processes (9).

Others have shown that embryos from women with endometriosis are six times more likely to show aberrant nuclear and cytoplasmic events compared to women without endometriosis (10). These events included cytoplasmic fragmentation, darkened cytoplasm and arrest before syngamy, reduced embryo cell numbers (7, 11, 12). Increased frequency of embryo arrest (7, 13), decreased implantation rates (7, 11, 14, 15) and early pregnancy loss (8, 16, 17, 18, 19, 20) are also present in women with endometriosis. Yet others have not found such anomalies (21, 22, 23, 24) and this topic remains controversial. Moreover, studies are not evident in the literature indicating which, if any, of these anomalies are heritable or span generations. Further impeding the issue, ethical values prohibit prospective, randomized clinical trials of human embryos and multigenerational embryo anomalies. Multigenerational trials are also subject to loss to follow up.

To define which anomalies actually lead to infertility and to assess multigenerational mechanisms underlying subfertility in endometriosis which are not accessible in women, we use an established endometriosis model in the rat (Endo) compared to controls (Shams) (25). We previously reported that ovaries of Endo rats have fewer antral follicles and corpora lutea and more luteinized unruptured follicles than Sham rats (26). Consistent with these observations, Endo rats ovulate fewer oocytes, develop fewer zygotes and at gestational day 15 have fewer viable fetuses and more pregnancy loss than Sham rats (26). In addition, Vernon MW and Wilson EA (25), the individuals who developed this endometriosis model, reported that the induction of endometriosis significantly reduced the number of pups at term by 48%.

We found developmental exposure to surgically induced endometriosis in rats (founders, F0 generation) caused morphological, structural and developmental anomalies in their oocytes and pre-implantation embryos (F1 Endo generation). Such anomalies include misaligned chromosomes, nuclear and cytoplasmic fragmentation, disorganized microtubule networks and delayed or arrested cleavage, which were not detected in F1 embryos from surgical controls (F0 Shams) (26). Interestingly, we have been able to restore endometriosis-associated infertility in this model with novel therapeutic approaches (27, 28). Some of these anomalies were found in F2 generation embryos born from F1 generation Endo rats that did not undergo surgery (26). Importantly, these anomalies are similar to those of embryos from women with endometriosis (9).

The rationale for our current study was that using a highly controlled endometriosis animal model (same species, strain, diet and environment) would eliminate confounding factors and novel multigenerational mechanisms of embryo loss would be discovered. Here we report for the first time that anomalous gene expression by F1 generation 8-cell stage embryos from F0 Endo rats compared to F0 Shams persists in the next generation 8-cell rat embryos (F2) born from F1 Endo rats but not from F1 Sham rats. Hence, our working hypothesis was that fetal exposure and fetal germ cell exposure to endometriosis in utero alters gene expression in first and second generation 8-cell stage embryos.

MATERIALS AND METHODS

Animal Care and the Induction of Endometriosis

The University of Missouri Institutional Animal Care and Use Committee, in accord with the National Research Council's Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press; 1996) approved all experiments. Sexually mature Sprague-Dawley female rats (72 days old, 225 g; Harlan, Madison, WS) were housed two per cage with ad libitum access to food and water on a 14L:10D cycle. After 14 days of environmental acclimation, females exhibiting regular 4 to 5 day estrous cycles, as determined by daily evaluation of vaginal cytology, were used for experiments.

Endometriosis was surgically-induced in rats (F0 Endo) as originally developed by Wilson and Vernon (25) and performed in our laboratory (26, 27, 28, 29) with slight modifications. Four equal size pieces (2×2 mm) from the distal two thirds of the left uterine horn were sutured to the arterial blood supply of the small intestine. The remaining rats underwent a control surgery with removal of the uterine horn and ovary (F0 Sham). All rats survived the surgeries. One week after surgery, daily evaluation of vaginal cytology was re-initiated to confirm normalcy of their reproductive cycle.

Collection and Evaluation of First (F1) and Second (F2) Generation Eight Cell Stage Embryos

Four weeks post-surgeries, when the endometriotic implants reach their maximal size (30, 31), cycles were synchronized (not super-ovulated) in F0 Endo (n=14) and F0 Sham (n=14) rats with a LHRH agonist (Sigma-Aldrich, St. Louis; 200 ug / 200 uL PBS per rat) to facilitate animal husbandry and management (26, 32). On the evening of the first proestrus after LHRH treatment (peri-ovulatory period) each female was placed into a cage with one proven breeder male. The next morning, evidence of sperm in vaginal lavages was designated as day 1 of gestation. Two Endo rats and two Sham rats failed to become pregnant after three exposures to different proven breeder males.

On gestational day 3, one-half of the F0 Endo (n=6) and F0 Sham (n=6) rats were euthanized by CO₂ asphyxiation and aortic transection. The oviduct and remaining uterine horn were excised and flushed with 0.5 mL PBS to collect F1 8-cell embryos. Two excellent quality sibling 8-cell stage embryos per pregnant rat were used for whole genome microarray analyses and for confirmation by quantitative real time PCR (qRT-PCR). Eight cell embryo quality was defined as morphological grade and blastomere cleavage rate using a stereoscope (Nikon SMZU, Nikon, Inc., Melville, NY). Excellent quality 8-cell embryos had eight symmetric blastomeres, no cytoplasmic fragmentation and no other anomalies. Lesser quality embryos had asymmetric cleavage and increasing numbers of cytoplasmic anomalies such as fragmentation, vacuoles, and darkening and were eliminated from further consideration.

The 8-cell stage of embryo development was chosen for analysis for several reasons. In the rat, the maternal to embryonic transition begins at the 2-cell stage and is completed by the 8-cell stage (33). Therefore, examining the 8-cell stage embryo would reveal the impact of endometriosis on the embryo gene expression rather than maternal gene expression. In addition, at the 8-cell stage, all blastomeres of the embryo are still considered equivalent and undifferentiated (34). As the goal of the study was to assess embryonic gene expression, the total number of embryos per rat was not quantified to facilitate more rapid processing of the first two high grade embryos discovered as not to compromise embryo RNA quality for the whole genomic microarray.

The remaining F0 Endo (n=6) and F0 Sham (n=6) rats were bred as described but allowed to gestate to term providing F1 pups. Following post-natal day 70, estrous cycle synchronization and breeding of the F1 females was performed as described for the F0 rats. On day 3, F1 rats were euthanized and excellent quality F2 8-cell embryos were collected as described.

Whole genome microarray analysis of F1 8-cell stage embryonic transcripts

RNA was isolated from two excellent quality 8-cell stage embryos per rat as per manufacturer's instructions using the PicoPure RNA Isolation kit (Molecular Devices, Sunnydale, CA), with the addition of DNase digestion on-column (Qiagen, Valencia, CA).

RNA was eluted from the column. For increased RNA yield, the eluted RNA was reapplied to the column and centrifuged at 13,000 g for 1 min.

First strand cDNA was synthesized using Illumina's TotalPrep Kit with addition of T7 Primer (San Diego, CA) and 70°C incubation for 5 min before addition of reverse transcriptase master mix. Immediately following, second strand cDNA was synthesized and purified with the Illumina TotalPrep Kit (San Diego, CA). A second round of amplification was performed using Ambion's MessageAmp II aRNA amplification Kit (Foster City, CA). Synthesis of aRNA was performed in a 40 µl unmodified reaction with 14 h incubation. Resulting aRNA was purified (Illumina TotalPrep Kit) and quantified on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). A second round of amplification was performed using the same procedures.

To synthesize biotin labeled cRNA, the Illumina TotalPrep RNA amplification kit was used. Quantity and quality of cRNA were assessed using a Nanodrop spectrophotometer (Thermoscientific) and gel electrophoresis. Biotin-labeled cRNA (750 ng) was hybridized to the Illumina RatRef12 Whole Genome Microarray Chip (22,523 genes, Illumina, San Diego, CA) by The University of Missouri DNA Core. For statistical analysis of microarray data, log₂ fold change was computed along with estimated log-odds ratios of differential expression as we previously described (35). A moderated t-test of the contrast Endo/Sham was conducted using the fitted model. Due to the large number of statistical tests, it was important to control properly the false discovery rate of differentially expressed genes via *p*-value adjustments using the method of Benjamini (36); resulting *p*-values are denoted by *q*. Only samples with a fold enrichment greater >1.90 and *q* < 0.15 were considered differentially expressed.

Genes of interest were selected from the microarray data using described statistical qualifications including DAVID Bioinformatics Resources 6.7 (Frederick, MD) pathway analysis (37, 38), biological relevance to known disrupted pathways in embryo development in endometriosis as evidenced in the literature (39) and by availability of primers/probes documented by the manufacturer to detect rat genes. Selection criteria for genes of interest included >1.90 fold enrichment, *P* < 0.005 and count > 10 genes as reported in the DAVID analyses.

Quantification of F1 and F2 8-cell embryonic transcript levels for genes of interest

Altered gene expression identified by microarray analysis was confirmed and quantified by qRT-PCR analyses. Only excellent quality 8-cell stage embryos (sibling embryos to those used for microarray) from the F1 generation were included. Two 8-cell stage embryos per rat from four different rats, randomly selected from the F1 lineage, provided analyses per surgery in quadruplicate. The experimental design for F2 rat embryos also included four rats per group with two excellent 8 cell embryos per rat. However, as fewer excellent quality 8-cell stage embryos were available, both good and excellent quality embryos were included from rats in the F2 Endo and F2 Sham lineages (Table 1).

RNA for qRT-PCR was isolated and amplified as described for the whole genome microarray analyses with the exception that the second round of amplification resulted in unlabeled aRNA. Second round aRNA (500 ng) was reverse transcribed using the Roche First Strand cDNA Synthesis kit for qRT-PCR (Indianapolis, IN). The qRT-PCR was performed in a 25 µl reaction volume that contained 1 µl of 10-fold diluted cDNA, 1 µl primer mix and 12.5 µl Sybr green master mix from SuperArray Bioscience Inc. (Bethesda, MD) and amplified in an ABI 7500 Smart Cycler (Applied Biosystems, Foster City, CA). The average Ct for the housekeeping gene, *B-actin* was 18.6 ± 0.22, and was not significantly different between samples (*P*=0.621).

Commercially available primers for genes of interest and an endogenous control gene β -actin (*ActB*) were purchased (SABiosciences Inc., Bethesda, MD). Changes in cycle threshold (ΔC_t) relative mRNA expression were calculated and statistical differences between Endo and Sham were calculated by comparing group mean ΔC_t . One-sided Student's t-Tests distinguished significant differences with $P < 0.05$ because directionality of the fold change was hypothesized based on the results from the microarray analysis.

RESULTS

Whole genome analysis of F1 8-cell stage embryo gene expression revealed several functional group clusters enriched in Endo embryos

Of more than 22,000 transcripts represented on the RatRef12 Whole Genome Microarray, 698 were differentially regulated in F1 Endo 8-cell stage embryos compared to F1 Sham embryos. Of these, 399 transcripts were up-regulated while 299 transcripts were down-regulated. After removing 31 transcripts lacking annotation and eliminating duplication among seven pairs of transcripts that mapped to the same genes, 600 genes remained for analyses (Suppl. Table 1). DAVID Bioinformatics Resources 6.7 tools revealed several statistically enriched terms, which included regulation of apoptosis, negative regulation of molecular function, cell cycle process, response to oxidative stress and RNA processing (Table 2).

The apoptosis pathway was chosen for further study due to the number of transcripts found to be different between F1 Endo and F1 Sham 8-cell stage embryos and the increased incidence of apoptosis reported in embryos from endometriosis mothers (9). The microarray analyses demonstrated differential expression between F1 8-cell stage embryos associated with regulation of apoptosis: apoptotic peptidase activating factor 1 (*Apaf1*); carbamoyl-phosphate synthetase 2 (*Cad*); Caspase 3 (*Casp3*); Diablo homolog (*Diablo*, also known as *Smac*); a DnaJ (Hsp40) homolog subfamily A member 3 (*Dnaja3*); nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (*Nfkb1a*); and poly (ADP-ribose) polymerase 1 (*Parp1*).

Confirmation of significant differential gene expression levels differences between F1 Endo and F1 Sham embryos by qRT-PCR analyses

Paralleling microarray data, qRT-PCR analyses (Fig. 1A, Table 3) showed F1 Endo 8-cell stage embryos expressed more *Diablo*, *Casp3*, *Parp1*, *Cad* and *Dnaja3*, less *Nfkb1a* and contrary to microarray results no statistical difference was found in *Apaf1* levels compared to F1 Sham 8-cell embryos. There was no difference between groups in *ActB* expression levels used to normalize expression of target genes ($P=0.172$).

Differential gene expression levels between F2 Endo and F2 Sham embryos by qRT-PCR analyses

Like microarray and qRT-PCR results from F1 8-cell embryos, levels of *Casp3* and *Cad* ($P = 0.01$) and *Parp1* and *Nfkb1a* ($P < 0.06$) were higher in F2 Endo than F2 Sham 8-cell embryos and *Apaf1* did not differ (Fig. 1B, Table 3). Unlike the F1 Endo and F1 Sham embryos, no differences were found. *ActB* expression levels did not differ between groups ($P=0.621$).

DISCUSSION

Developmental exposure to endometriosis causes differential gene expression in F1 and F2 generation 8-cell stage embryos

We report here for the first time differential gene expression by excellent quality 8-cell stage embryos from two generations (F1 and F2) from an in vivo endometriosis model (F0 Endo) but not controls (F0 Sham). Excellent morphological quality F1 8-cell stage embryos from this endometriosis model differentially expressed 698 distinct genes from over 22,000 genes represented on a whole genome microarray compared to F1 Sham embryos. This is more evidence that assessing morphology alone is insufficient to predict embryo quality and fecundity. Future studies of gene expression in lesser morphological quality embryos may provide further insights into the expression of more or different genes associated the mechanism of embryo demise in endometriosis.

Differential gene expression in several pathways relevant to regulation of apoptosis, negative regulation of molecular function, cell cycle process, response to oxidative stress and RNA processing during embryo growth, development and demise in endometriosis were identified. This could reflect the slower development we reported previously in Endo embryos (26). Delayed cleavage is another commonly found embryonic anomaly in embryos from women with endometriosis (9) supported by our observation of differential expression of cell cycle regulators.

Apoptosis Pathways in 8-Cell Embryos of the F1 and F2 Generations

A significant proportion of genes in F1 Endo 8-cell stage embryos, maternally exposed to endometriosis in utero (F0 mothers) were associated with the apoptosis regulatory pathway. Further, a subset of these anomalies was also expressed by F2 8-cell stage embryos born from F1 Endo rats and exposed to endometriosis as fetal germ cells, but not from Sham rats. These data help support our hypothesis that developmental programming for cell death in morphologically normal appearing F1 and F2 8-cell stage embryos may contribute to subfertility in endometriosis. There is increased apoptosis in embryos from women with endometriosis (10), and endometriosis has a familial component (2, 4). Yet the mechanisms causing embryo apoptosis in several generations in women have not been elucidated. Further studies with this model may help fill this gap in our knowledge.

Caspase 3 (*Casp3*) gene expression was upregulated in both F1 Endo and F2 Endo embryos compared to the F1 and F2 Sham embryos, respectively. Increased expression of *Casp3* mRNA has been considered a potential marker of poor embryo quality in the bovine, although expression levels fluctuated widely between embryos (40). The CASP3 enzyme is an effector of the caspase dependent apoptosis cascade (41). The mechanism of action for CASP3 has been well-studied. CASP3 works to cleave directly many substances including the structural and regulatory proteins in the cell nucleus, cytoplasm and cytoskeleton (42). This leads to elimination of key factors needed for survival and subsequently cell death. CASP3 also cleaves several DNA repair molecules including PARP1 (42). Hence, increased *Casp3* transcripts in F1 and F2 Endo lineage embryos may be part of a mechanism decreasing embryo quality and increasing in programmed cell death.

Poly(ADP-ribosyl)ation polymerase (*Parp1*) is a DNA damage sensor and cytotoxic mediator (43). Eight-cell embryos from F1 Endo rats had 10 fold more *Parp1*, suggesting that they were in distress. High levels of *Parp1* are associated with the need for DNA repair (43). *Parp1* can also regulate chromatin structure binding the linker histone, H1 (44), which permits easier access to the promoter region by transcription factors leading to increased expression (44). DNMT1 expression is regulated by PARP1 binding and the presence of

PARP1 provides protection from silencing DNA methylation (43). It is possible that the increased amounts CASP3 enzyme in Endo rat 8-cell embryos are cleaving *Parp1* thus rendering it less effective for DNA repair.

Besides serving as a cytotoxic mediator, *Parp1* is also involved in transcriptional regulation. Most notably *Parp1* overexpression has been associated with modulating the function of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (43) and subsequent expression of inflammatory mediators. It is interesting to note that oxidative stress, which was a pathway identified in the DAVID analysis, which could be a potential mechanism of endometriosis mediated embryo loss, has been shown to cause DNA damage and activate PARP1 (44). *Parp1* overexpression in F1 Endo embryos may therefore be a direct result of oxidative stress from the endometriosis environment leading to increased embryo death. A role for *Parp1* in F2 Endo embryos merits further investigation with additional embryos as it was nearly significant in these studies ($P=0.055$).

Like PARP1, carbamoyl-phosphate synthetase 2 (CAD) has been shown to be degraded by CASP3 during the caspase-dependent apoptosis cascade (45). The CAD enzyme, involved in pyrimidine biosynthesis, is upregulated in F1 and F2 Endo rat embryos. Considered a checkpoint in RNA and DNA synthesis, CAD is an indicator of proliferation although excess *Cad* expression has been associated with apoptosis (45). Upregulation of *Cad* in F1 and F2 Endo embryos could indicate an imbalance in the pyrimidine ribonucleotides synthesized as well as a feedback mechanism trying to rescue the embryo from death. This ribonucleotide imbalance could initiate or contribute to apoptosis and be specifically targeted during apoptosis (45).

Increased expression of the Diablo homolog (*Diablo*), which contributes to apoptosis, was noted in F1 Endo embryos compared to F1 Sham controls. During apoptosis DIABLO is released into the cytosol where it removes inhibitors of CASP 3 and 9 action (46). Apoptosis induced by cAMP requires Smac/DIABLO transcriptional up-regulation (46). Hence, elevated *Diablo* expression may explain amplified expression of *Casp3* and thereby apoptosis in F1 Endo embryos.

Interestingly, *Diablo* mRNA expression is enhanced in kidney cells undergoing tumor necrosis factor (TNF)-induced apoptosis (47). As TNFα is elevated in peritoneal fluid (PF) of women with endometriosis (48), this may be part of a mechanism increasing *Diablo* in F1 embryos, which were directly exposed to inflammatory endometriotic PF entering the oviduct. Other mechanisms may also be involved as *Diablo* was not elevated in F2 Endo embryos but *Casp3* expression was greatly elevated. The absence of direct exposure of the F2 Endo embryos to endometriotic PF (level of exposure was as germ cells) provides a plausible explanation why *Diablo* was not elevated in F2 Endo embryos.

Dnaja3 is a mitochondrial chaperone protein that induces mitochondrial fragmentation and apoptosis when protein levels are elevated (49). Overexpression of *Dnaja3* mRNA disrupts transport of heat shock protein 70 and causes apoptosis (49). *Diablo* and *Dnaja3* were not upregulated in F2 embryos, which could indicate that germ cell exposure to endometriosis may only reprogram expression of select genes.

Nfkb1a mRNA expression was decreased in F1 Endo 8-cell stage embryos compared to controls. NFKBIA acts as an inhibitor of the transcription factor Nuclear Factor Kappa B (NF-κB) (50). When NF-κB is bound to NFKBIA it is inactive and unable to enter the nucleus. With a decrease in the inhibitor of NF-κB, there is a potential increase in the level of active (unbound) NF-κB that can enter the nucleus and cause transcription of anti-apoptosis genes (50). Down-regulation of *Nfkb1a* was not found in F2 embryos, which may

again indicate germ cell exposure to endometriosis may only reprogram expression of select genes. Others have also found NF- κ B regulation anomalies associated with endometriosis (51).

Reactive oxygen species were implicated as a potential source of endometriosis-related infertility (52). Higher levels of reactive oxygen species (ROS) and lipid peroxidases in PF from women with endometriosis, (53, 54) may account for genes associated with the response to oxidative stress being actively transcribed in the Endo 8-cell embryos. Transcripts associated with RNA processing were enriched in Endo embryos suggesting that the embryos could be attempting to respond to a stressor such as the before mentioned oxidative stress.

In summary, this is the first report to describe differential gene expression by F1 8-cell embryos developmentally exposed to endometriosis in utero and by F2 8-cell embryos exposed to endometriosis as fetal germ cells. The outcome therefore supports our hypothesis that developmental exposure to endometriotic lesions causes differences in embryonic gene expression across two generations offering a possible explanation for the familial clustering of endometriosis.

There are limitations associated with this study. Future studies with larger sample sizes are needed to facilitate obtaining sufficient amounts of embryo RNA from future generations, which in this study may have contributed the failure to detect expression in some of the genes detected in the F1 microarray and qRT-PCR analyses but not in the F2 generation. Alternatively, these gene expression differences may be true differences, as not all genes may be affected across generations. Studies of the F3 the generation will determine if these are truly transgenerational effects, as the F3 generation would not have experienced any developmental exposure to endometriosis. Moreover, while others and we have historically reported decreased litter size and pregnancy loss using this model (25, 26), it will be important to determine whether the numbers of embryos and pups of all future generations are declining. This research examined F1 and F2 8-cell stage embryos and did not generate F2 pups.

Identification of specific genes, gene mutations or alterations in gene expression to determine success or failure of 8-cell stage embryo health and viability would be of significant clinical value for women with endometriosis and infertility. Using in vitro fertilization and embryo development, 8-cell stage embryos may undergo blastomere biopsy with pre-implantation screening to detect endometriosis specific anomalies prior to embryo transfer. To date, we would propose a panel of gene expression screening markers beginning with members of the apoptosis pathway Casp3, Parp1 and Cad and adding new markers as future studies detect additional aberrant multigenerational gene expression markers in other pathways identified by microarray analyses in these studies. In conclusion, understanding the perplexing multiplicities of familial inheritance and infertility by defining and distinguishing cellular and molecular mechanisms responsible for endometriosis- will provide insights into novel therapeutic approaches for endometriosis-associated infertility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Expression of apoptosis-related genes of interest in F1 and F2 generation 8-cell embryos identified by qRT-PCR

A. F1 Endo embryos had increased expression of Casp3*, Parp1*, Cad*, and Dnaja3*. Nfkb1a* expression was decreased in F1 embryos from rats with endometriosis. B. Four of seven differentially regulated genes in F1 generation 8-cell stage embryos were also differentially expressed in the F2 8-cell stage embryos. F2 generation Endo rats had increased mRNA expression of Casp3*, Parp1†, Cad* and Nfkb1a†. (Fold change in gene expression is reported relative to ActB expression. Data are shown as mean ± SEM.

Table 1
Samples for F2 8-cell stage embryos analyzed by Q-PCR. Each sample contains two embryos

Gene	P value	F2 Endo		F2 Sham	
		# Samples	# Siblings	# Samples	# Siblings
Diablo	0.215	4	4	3	2
Casp3	0.016*	4	4	3	2
Parp1	0.055**	5	4	5	2 sets of 2
Cad	0.010*	4	4	3	2
Nfkbia	0.056**	4	3	3	2
Dnaj3	0.288	4	4	3	2
Apaf1	0.478	4	3	2	0

* Endo > Sham, P<0.05

** Endo > Sham, P 0.06

Table 2
GO Term DAVID pathway analysis of differentially expressed genes from F1 Endo F1
versus F1 Sham 8-cell embryos

Term Inclusion Criteria: Gene count 10; Fold enrichment 1.90; P value 0.005.

Go Term	Number of Genes	Fold Enrichment	P-value
Apoptosis Related Pathways			
Cell death GO:0008219	29	2.04	0.0005
Death GO:0016265	29	2.00	0.0006
Apoptosis GO:0006915	25	1.96	0.0021
Programmed cell death GO:0009719	25	1.93	0.0026
Other Pathways			
Negative regular molecular function GO:0044092	23	2.25	0.0006
Cell cycle process GO:0022402	24	1.94	0.0030
Response to oxidative stress GO:0006979	15	2.43	0.0034
RNA processing GO:0006396	23	1.94	0.0038

Table 3
Fold change in gene transcripts between endometriosis versus sham 8-cell embryos from F1 and F2 generations

Genes	Microarray		QPCR		QPCR	
	F1	F2	F1	F2	F1	F2
Diablo	3.82	3.20	5.29	27.47	0.009	0.002
Casp3	3.06	3.02	10.46	13.03	0.003	0.009
Parp1	3.02	4.76	13.03	-8.98	0.009	0.040
Cad	3.02	1.99	13.03	2.85	0.009	0.034
Nfkb	3.02	3.60	13.03	-2.24	0.009	0.210
Dnajl3	3.02	3.60	13.03	1.06	0.009	0.487
Apaf1	3.02	3.60	13.03	1.06	0.009	0.487