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The Role of the Retinoic Acid Pathway in Decidualization of Endometrial Stromal Cells

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Abstract: *This study found RBP1 levels to be reduced in endometriosis lesions and in ME-SFCs derived from endometriosis subjects. In addition to endometriosis lesions, RBP1 levels have been found to be reduced in breast cancer, ovarian cancer, cervical, and endometrial cancer. The reduction of RBP1 in cancer suggests that RBP1 may act as a tumor suppressor (Mendoza-Rodriguez, et al. 2013). In fact, in a study of breast cancer cell lines over- expressing RBP1 led to reduced survival of breast cancer cells in suspension by inhibiting anchorage independent growth mediated by the PI3K/AKT. The PI3K/AKT has been shown to be elevated in endometriosis lesions compared to normal eutopic endometrium of control subjects, however, this is thought to contribute to the decidualization defects observed in endometriosis-associated infertility. It is, however, interesting to make a comparison between breast cancer and endometriosis. In breast cancer higher levels of RBP1 reduced p-AKT/AKT levels, thereby inhibiting the PI3K/AKT pathway leading to reduced survival of cells in suspension cultures; this is thought to be due to altered levels of retinoic acid. Endometriosis lesions have reduced expression of RBP1 and higher levels of pAKT/AKT that is not reduced upon decidualization. RBP1 expression and the PI3K/AKT pathway in endometriosis have not yet been connected in the same way that they are connected in breast cancer. In our future studies we should focus on the other potential roles that RBP1 could play in the development of endometriosis rather than solely investigating its effect on decidualization.*

Keywords: retinoic acid pathway, decidualization, endometriosis, infertility

Introduction:

Retinoic acid is an important mediator of organ development, cell differentiation, proliferation, and apoptosis in many tissues (Jiang, et al. 2018). It is synthesized from dietary retinoids as carotenoid and retinyl ester or from circulating retinol bound to RBP4 (Jiang, et al. 2018). Retinyl esters and carotenoids enter endometrial stromal cells after being released from chylomicrons, while RBP4 bound retinol entry is mediated by STRA6 (Jiang, et al. 2018). Once inside the cell retinyl esters can either be in the storage form of retinoids where retinol is converted to a retinyl ester by lecithin: retinol acyltransferase (LRAT) or retinyl esters can be stored as retinol after conversion with retinyl ester hydrolase (REH) (Jiang, et al. 2018). Inside the cell retinol is bound by retinol binding protein 1 (RBP1). RBP1 protects retinol from catabolism or metabolism while also serving as the preferred substrate for enzymes that convert retinol to retinyl esters for storage or to retinal for retinoic acid production (Jiang, et al. 2018; Napoli 2016). RBP1 knockout mice exhibit reduced retinoic acid production (Pierzchalski, et al. 2014a). The enzymes involved in the conversion of retinol to retinal and retinal to retinoic acid are aldehyde dehydrogenases, including ALDH1A1. The retinoic acid produced by these enzymatic reactions is then transported to the nucleus by cellular retinoic acid binding protein 2 (CRABP2) or fatty acid binding protein 5 (FABP5), leading to two opposing signaling cascades for apoptosis/differentiation and proliferation, respectively.

Retinoic acid transported to the nucleus by CRABP2 binds to retinoic acid receptors (e.g. RARα, RARβ, and RARγ), which heterodimerize with retinoid x receptors (RXRs) and associate with retinoic acid receptor response elements leading to gene transcription that induces pro-apoptotic, pro-differentiation, and anti-proliferative effects. Retinoic acid transported to the nucleus by FABP5 binds to peroxisome proliferator-activated receptor β/δ (PPARβ/δ), which also heterodimerizes with RXRs and associates with peroxisome proliferator response elements (PPREs) to promote the transcription of genes that induce anti-apoptotic and pro-proliferative effects. It has also been shown that endometriotic stromal cells from endometriosis subjects have lower levels of CRABP2 and higher levels of FABP5 and that a low CRABP2:FABP5 ratio leads to stromal cell protection from apoptosis and a pro-proliferative state (Pavone, et al. 2010).

In addition, CRABP2 and FABP5 have been shown by Pavone et.al. to exert opposing effects on decidualization, the process by which stromal cells in the endometrium differentiate during the mid-secretory phase to promote uterine changes required for embryo implantation and placental development (Burney and Giudice 2012). Lower levels of CRABP2 and higher levels of FABP5 not only promoted survival in endometrial stromal cells, but also were found to reduce in-vitro decidualization. If these findings were replicated in eutopic endometrium of women with endometriosis, they might account for some of the endometriosis-associated infertility due to decidualizaiton defects. These are interesting findings because up until this point, retinoic acid was thought to inhibit decidualization. It has been proposed that the effect of retinoic acid on decidualization may be dependent on which signaling pathway is more prominent in the stromal cells (via CRABP2 or via FABP5) (Pavone,

et al. 2017). Additionally, studies employing rodent models have demonstrated that retinoic acid receptors and proteins involved in retinoic acid synthesis are regulated by estrogen and progesterone throughout the menstrual cycle lending support to the idea that retinoic acid plays a role in the development of a receptive endometrium (Deng, et al. 2003; Ma, et al. 2012; Ohno, et al. 2008; Ozaki, et al. 2017). Further work needs to be done to characterize retinoic acid synthesis and the signaling molecules in the eutopic endometrium of women with and without endometriosis to better understand what role retinoic acid may play in the decidualization defects associated with endometriosis.

In past literature the most highly differentially expressed gene between control and endometriosis subjects' ME-SFCs was *ADLH1A1*, which encodes an aldehyde dehydrogenase involved in the synthesis of retinoic acid from retinol. *ALDH1A1* was significantly reduced in both vehicle-treated and cAMP-stimulated ME- SFCs from endometriosis patients when compared to controls. We propose that dysregulation of the retinoic acid pathway in the setting of endometriosis contributes to the decudalization defects observed in ME-SFCs from endometriosis patients when compared to ME-SFCs from controls and therefore, in this study we investigate the role of retinoic acid pathway genes in in-vitro decidualization and endometriosis.

Aims:

1. Determine if retinoic acid pathway genes, including *ALDH1A1* exhibit altered expression in ME-SFCs during in vitro decidualization
2. Determine if additional retinoic acid pathway genes are differentially regulated in endometriosis ME-SFCs compared to control ME-SFCs
3. Determine if knocking down retinoic acid pathway genes alters decidualization capacity

Methods:

RT^2 Profiler of retinoic acid pathway signaling:

Four (n=4) control and n=4 endometriosis subjects' ME-SFCs were grown to confluence in 6 well plates at passage 2 and then stimulated with either decidualization media ± 8-Br-cAMP (cAMP, 0.5mM, Sigma) or an equivalent volume of PBS (vehicle) for 6hrs. Decidualization media contained 2% MSC FBS (Gibco Cat# 12662029), 1x glutamine (Gibco Cat# 25030081), 1% penicillin -streptomycin (Gibco Cat# 15140122), and 100ug/ml normocin (Invivogen Cat# ant-nr-1) in phenol red free DMEM (GIBCO Cat# 21063029). After 6hrs of stimulation with vehicle or cAMP cells were placed on ice and washed with cold 1X PBS, aspirated dry, and harvested in 600ul/well mirVANA lysis buffer (Invitrogen), which was stored at -80°C until the RNA was isolated. Total RNA was collected using the mirVANA miRNA isolation kit (Invitrogen AM1561) and DNA contaminants were digested with DNA-*Free* DNAse removal kit (Invitrogen AM1906). RNA concentration and quality were measured using the NanoDrop. First Strand cDNA Synthesis for the RT^2 Profiler was performed using the RT^2 First Strand Kit (50) (Qiagen Cat#330404). Genomic DNA (gDNA) elimination was first performed by adding 4uL of Buffer GE to 800ng RNA in a total volume of 20uL and then incubating the samples at 42°C for 5min. Samples were immediately placed on ice and the reverse transcription mix was added according to company protocols. RNA was converted to cDNA using Qiagen First Strand Synthesis kit using a Gene AMP PCR System 9700 (Applied Biosystems), according to the following protocol: 42°C for 15min followed by 95°C for 5min. 182uL RNAse free water was then added to dilute sample for use in the RT^2 Profiler Arrays and samples were stored at -20°C. 102uL of each cDNA sample was then separately added to 650uL RT^2 SYBR Green ROX qPCR Mastermix (Qiagen Cat#330523) and 548uL RNase-free water and placed in a RT^2 PCR Array Loading Reservoir (Qiagen Cat# 338162) from which each mixture was added to the RT^2 Profiler Array for Retinoic Acid Signaling plate (Qiagen Cat#PAHS-180ZG-4), according to the manufacturer's suggested plate map. RT^2 Profiler Array was run on the Roche Light Cycler 480 qPCR machine under the following conditions: 1 cycle: 10min at 95°C, followed by 45 cycles of 15sec at 95°C then 1min at 60°C. Data was analyzed using the Qiagen Data Analysis Center for the RT^2 Profiler PCR Array for Retinoic Acidsignaling.

Confirmation of RBP1 gene expression by qPCR using a larger sample size:

ME-SFCs isolated from ME collected from 7 control and 7 endometriosis subjects were grown, stimulated, and harvested for RNA isolation, as described in methods for "*RT^2 Profiler of retinoic acid pathway signaling"* above. The RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Cat# 4368814) with an

RNAse inhibitor (Applied Biosystems Cat# N8080119) using the following protocol on the Verti PCR machine: 25°C for 10min, 37°C for 120min, 85°C for 5min. Samples were centrifuged, diluted 10x in RNAse free water, and stored at - 20°C until use. qPCR was performed using Taqman Gene Expression Master mix (Applied Biosystems Cat# 43-690-16) and Taqman qPCR Assays for *GAPDH* (Assay# Hs99999905_m1) and *RBP1* (Assay# Hs01011512_g1) on the Viia7 qPCR Machine using the protocol: 50°C for 2min, 95°C for 10min, and 45 cycles of: 95°C for 15s followed by 60°C for 1min. qPCR data was analyzed using the delta delta CT method with *GAPDH* as the housekeeping gene. Statistical analyses were done using the Mann Whitney Test in Graph Pad Prism 5.

Decidualization time course of ALDH1A1 and RBP1 gene expression:

ME-SFCs from 5 control subjects were grown to confluence at passage 2 and stimulated with vehicle (PBS + decidualization media) or 0.5mM 8-Br-cAMP in decidualization media for 6, 24, and 48hrs. At each time point cells were harvested and RNA was isolated for each subject, as described in the methods of "*RT*^{2} *Profiler of retinoic acid pathway signaling"* above. qPCR was performed as described in *"Confirmation of RBP1 gene expression by qPCR in a larger sample size"* using *GAPDH* (Assay#Hs99999901_m1) as the housekeeping and *RBP1 (*Assay# Hs01011512_g1) and *ALDH1A1*(Hs00946916_m1) as the target genes. qPCR data was analyzed using the delta delta CT method with GAPDH as the house keeping gene. Statistical analysis was done using a two-way ANOVA with a Bonferroni Post-Hoc test to compare vehicle and cAMP stimulation over time and within a time point in Graph Pad Prism 5.

RBP1 knockdown experiments using ME-derived SFCs:

On-Target Plus Non-Targeting siRNA pool (Dharmacon Cat#L-003300-00-0005) and RBP1 siRNA pool (Dharmacon Cat# D-001810-10-05) delivered as 5nmol lyophilized powders were prepared by adding 50uL 1x siRNA Buffer (prepared from 5X siRNA buffer (Dharmacon Cat#B-002000-UB-100, diluted in RNase free water) and gently shaken on an orbital mixer for 30min. siRNA was then analyzed on the NanoDrop Spectrophotometer 2000 (Thermo Scientific) to verify the concentration.

ME-SFCs from 6 control subjects P2 to P4 were grown to confluence in 6 well plates. Each subject's cells were treated in duplicate (2 wells of a 6 well plate) with either Dharmafect 1 transfection reagent (Dharmacon Cat#T-2001-01) 3.5ul/well alone, 25nM non-targeting siRNA+Dharmafect 1, or 25nM RPB1 siRNA+Dharmafect 1. The transfection reagent volumes are depicted in Table 1. According to the Dharmafect protocol, Dharmafect 1 and 5uM siRNA were separately added to serum-free medium (DMEM, Gibco Cat#11995081) and incubated at room temperature for 5min. The Dharmafect 1 solution and siRNA solutions were then mixed together and incubated at room temperature for 20min. The Dharmafect 1-siRNA solution was then added to antibiotic-free complete knockdown medium (DMEM (Gibco Cat#11995081) containing10% MSC FBS, Gibco Cat#12662011 and 1XGlutamine (Gibco Cat#25030-081)). Cells were washed once with 1XPBS to remove antibiotics for old cell culture medium and medium was replaced with the knockdown medium for either the non-targeting siRNA, RBP1 siRNA, or Dharmafect alone which was the same protocol without the addition of siRNA.

Table 1 1X siRNA Transfection Reagent Volumes

			Tube 1		Tube 2			
Plating Format (wells/plate)	Surface area (cm2/well)	Final Concentration of siRNA (nM)	Volume of 5uM siRNA (ul)	serum-free medium (uL)	Volume of Dharmafect reagent (uL)	Serum-free Medium (uL)	Complete Medium (uL/well)	Total Transection Volume (uL/well)
6	10	25	10	190	3.5	196.5	1600	2000

Cells were incubated in the knockdown medium for 48hrs at 37°C and 5%CO_2, at which point one well from each condition (Dharmafect alone, non-targeting siRNA, and RBP1 siRNA) were changed to vehicle decidualization medium described in "*RT2 Profiler of retinoic acid pathway signaling"* and one well from each condition was changed to cAMP decidualization medium "RT2 Profiler of retinoic acid pathway signaling" and incubated for and additional 24hrs at 37°C and 5%CO_2. At the end of this 24hr incubation, plates were centrifuge at 1500rpm, 4°C, for 10min in Sorvall RT 6000D Centrifuge (Dupont) and then placed on ice for the collection of supernatants and cell lysates. Supernatants were collected and diluted to determine

IGFBP1 concentrations using the IGFBP1 ELISA (R&D Systems Duoset, Cat#DY871). Left over supernatants were aliquoted and stored at -80°C. Once supernatants were collected, cells were washed twice with cold 1XPBS on ice and collected in 50-100uL RIPA Buffer containing 50mM Tris pH7.4, 100mM NaCl, 0.5% TritonX-100, 1mM sodium orthovanadate, 2mM sodium fluoride, 0.5% deoxycholic acid, and 1X Halt Protease Inhibitor (Thermo Scientific Cat#78430). RIPA lysates were stored at -80°C until processed by pipetting up and down 20 times, vortex mixing 30s on high, and centrifuging in cold room at max speed (14,000 rpm) in Eppendorf 5415C centrifuge.

Results:

Retinoic acid pathway genes are altered during early in-vitro decidualization.

Six hrs post-cAMP stimulation *EGR1, TUBB3, MYC, CDX1, and FABP5* mRNAs were significantly down-regulated in control ME-SFCs when compared to vehicle stimulation, while *WNT5a* and *HOXA5* mRNAs were significantly up-regulated in control ME-SFCs following cAMP stimulation (Figure. 1*). GLI1, DHRS9, SOX9, and CY1P1B1* were down-regulated and *HOXB1* was up-regulated in cAMP stimulated control ME-SFCs when compared to vehicle-treated control ME-SFCs; however, the differences were not statistically significant (Figure. 1.). ME-SFCs from endometriosis subjects stimulated with cAMP for 6hrs showed similar differential expression patterns in genes related to the retinoic acid pathway; however, the gene differences that reached statistical significance differed from the control subjects (Figure. 2).

Retinoic acid pathway genes are differentially expressed by control ME-SFCs during decidualization

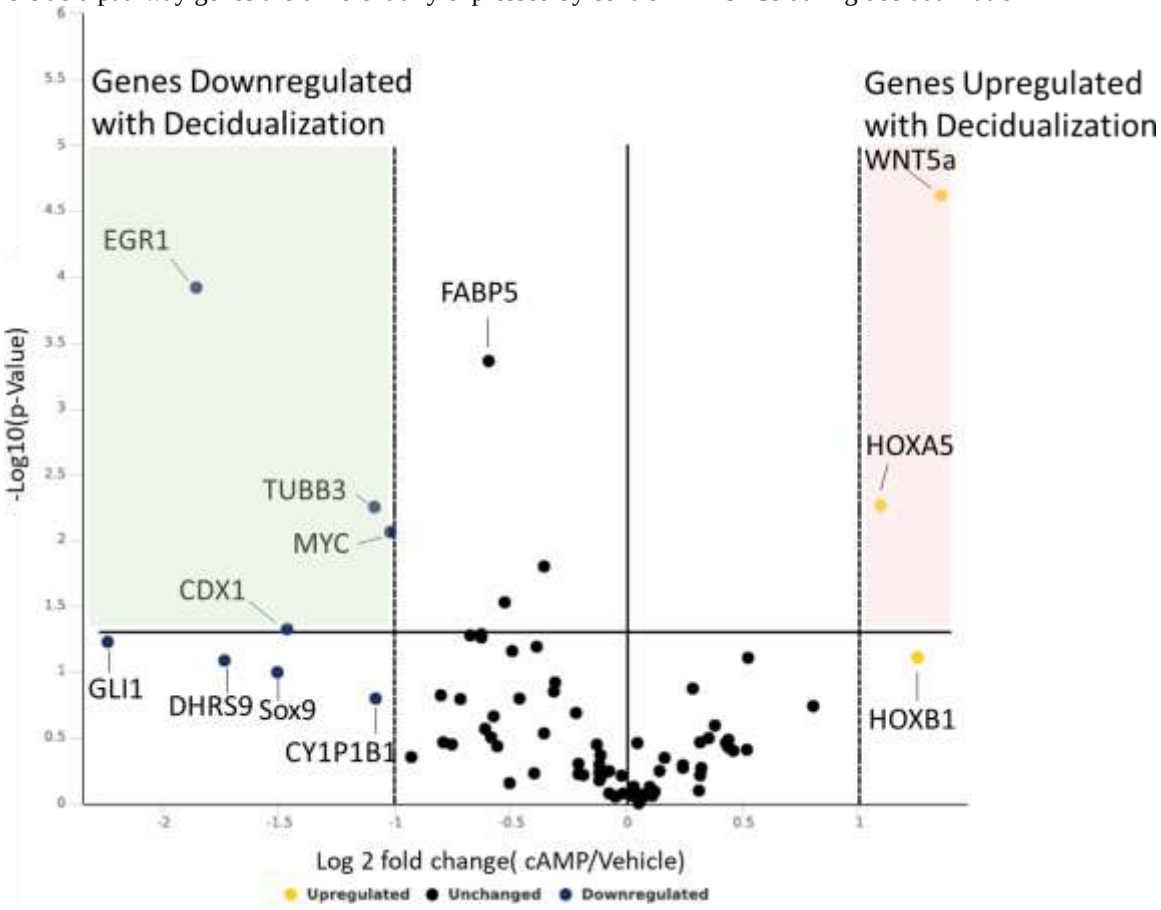

Figure 1. Volcano plot of retinoic acid signaling pathway genes altered by cAMP stimulation of control ME-SFCs found using the retinoic acid signaling RT^2 profiler. N=4

GLI1 and *SOX9* were significantly down-regulated and *WNT5*a and *HOXB1* were significantly up-regulated in decidualizing ME-SFCs obtained from endometriosis subjects (Figure. 2). *EGR1, DHRS9, CDX1,* and *GBX2* were down-regulated and *CD38* and *RBP2* were up-regulated in endometriosis ME-SFCs exposed to cAMP for 6hrs; although, not significantly (Figure

2).

Retinoic acid pathway genes are differentially expressed by endometriosis ME-SFCs during decidualization

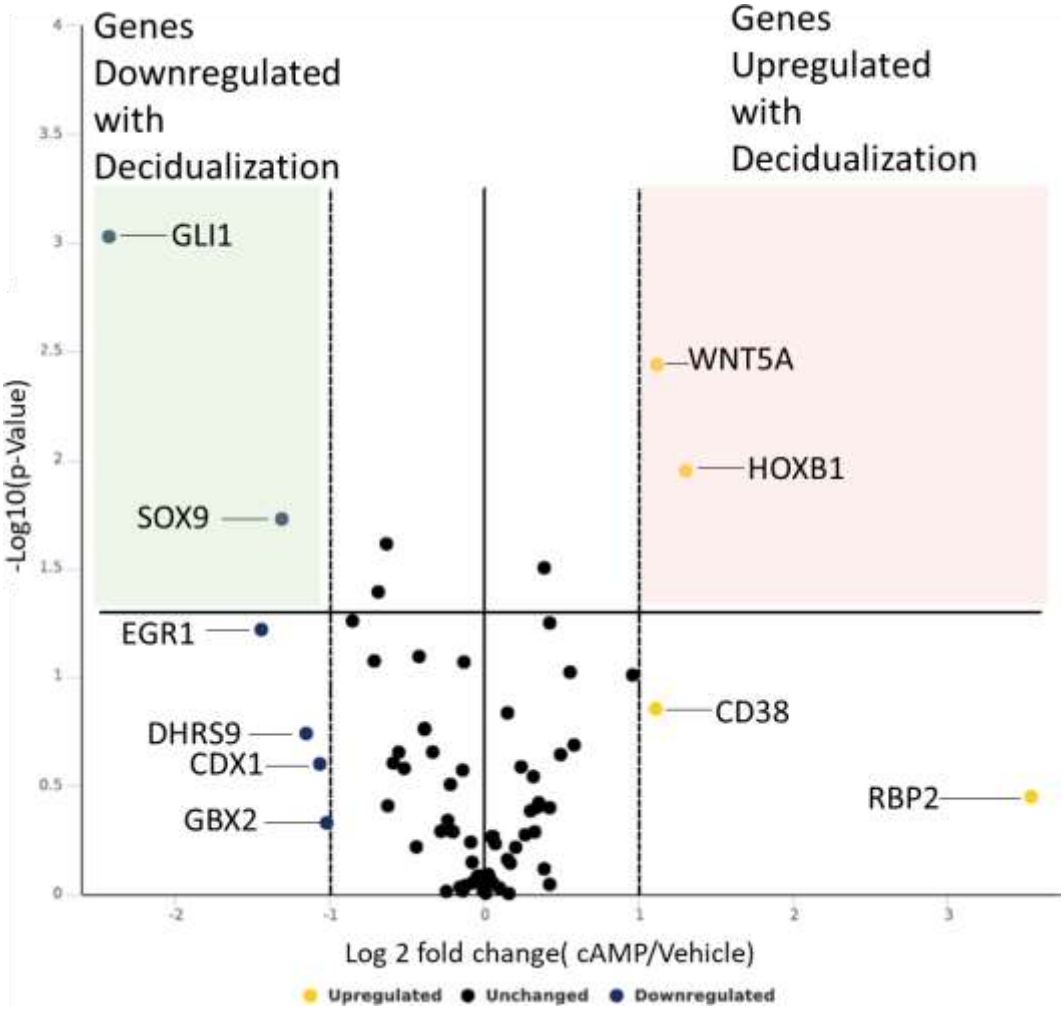

Figure 2. Volcano plot of retinoic acid signaling pathway genes altered by cAMP stimulation of endometriosis ME-SFCs found using the retinoic acid signaling RT^2 profiler. N=4

Retinoic acid pathway genes are differentially expressed in endometriosis and control ME-SFCs

In addition to assessing the changes in the retinoic acid pathway due to decidualization, we investigated whether this pathway was dysregulated in ME-SFCs obtained from endometriosis subjects after vehicle vs. cAMP stimulation. Under vehicle conditions endometriosis subjects' ME-SFCs were found to have significantly reduced expression of *ALDH1A1* and *EGR1* compared to controls (treated under vehicle conditions) and reduced levels of a gene of interest, *RBP1* p=0.09 (not statistically significant) (Figures 3a and 3b). The expression of *DHRS3*, *RARRES3*, and *RBP4* were all reduced, but not significantly, while *GBX2* mRNA expression was increased non-significantly in vehicle- treated ME-SFCs from endometriosis subjects compared to controls (Figure 3a).
Interestingly, *GBX2* expression was increased in vehicle-treated ME-SFCs from endometriosis subjects compared to controls and downregulated in cAMP-stimulated cells vs. vehicle-treated cells (Figures 2 & Figure 3a). Only one gene was significantly differentially regulated in ME-SFCs from control and endometriosis subjects stimulated with cAMP (Figure 4). *ALDH1A1* expression was significantly lower in endometriosis subjects' ME-SFCs exposed to cAMP compared to control subjects' ME-SFCs exposed to cAMP (Figures 4a and 4b). The expression of *RBP1*, *DHRS3*, *RARRES3*, and *RBP4* were again all lower in endometriosis subjects' ME-SFCs exposed to cAMP compared to controls' ME-SFCs; however, this

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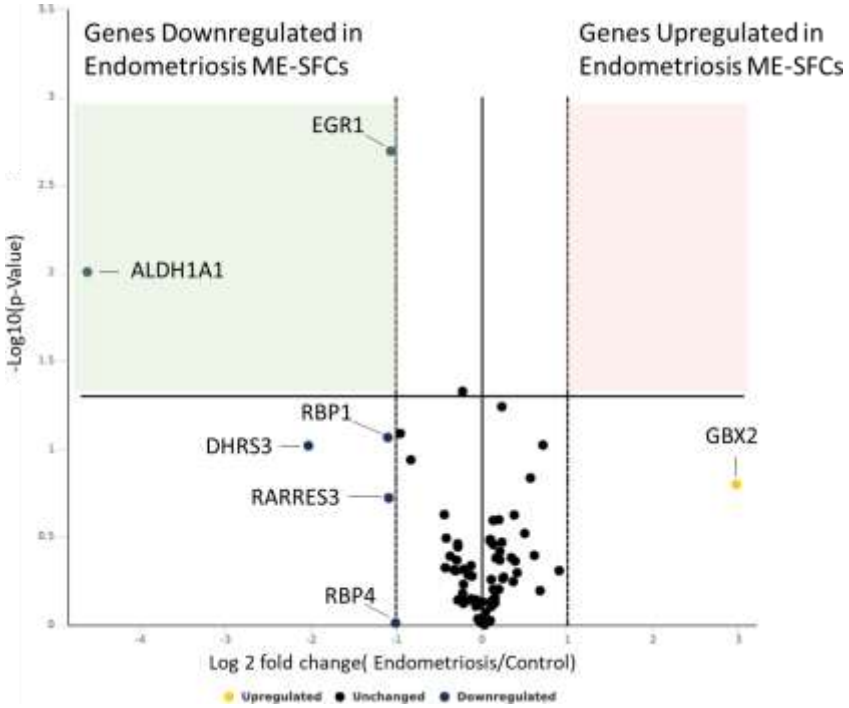
reduction was not statistically significant. Interestingly, *RBP1* reduction approached significance (p=0.06, Fig. 4a and 4b). The expression of *CYP1B1*, *GBX2*, and *RBP2* were all increased in endometriosis subjects' ME-SFCs, but did not reach statistical significance (Fig. 4a).

Differentially Regulated Genes in Vehicle Stimulated Endometriosis Vs Control Samples

a.



b.

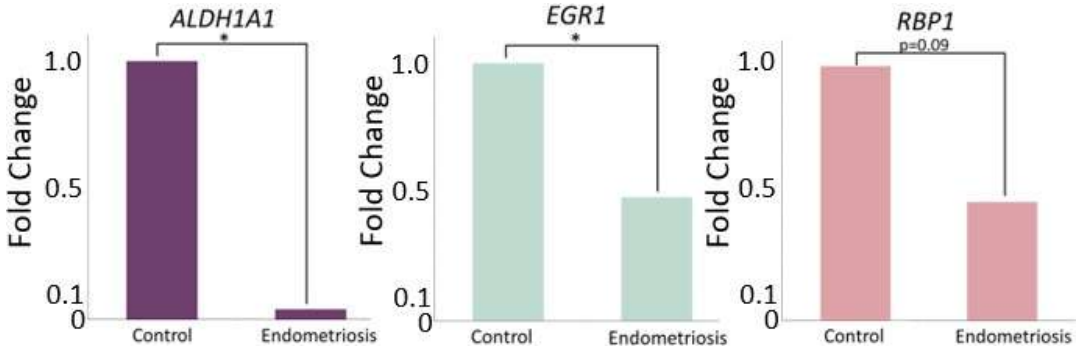
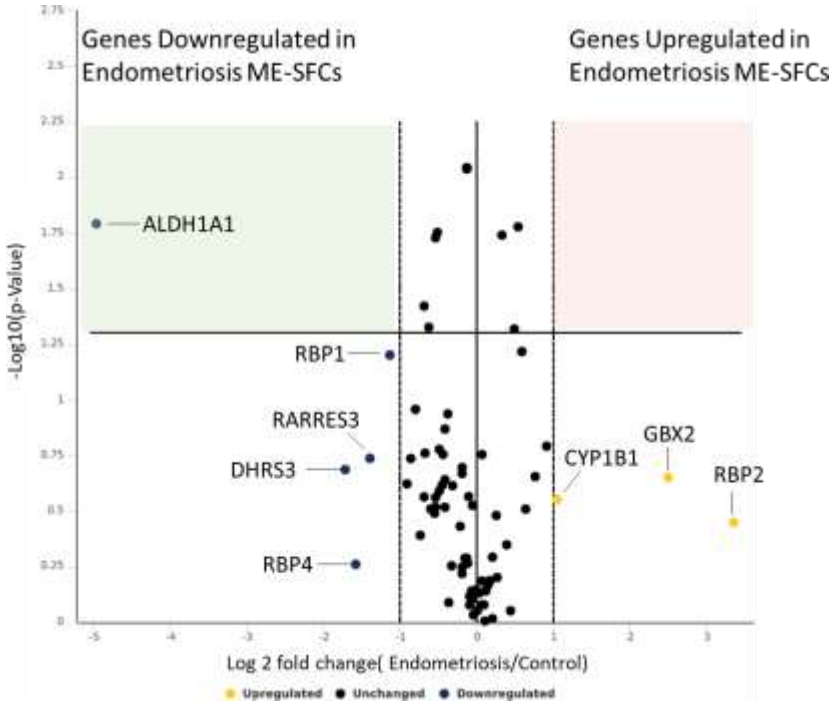


Figure 3. Differential expression of retinoic acid signaling pathway genes comparing ME-SFCs obtained from endometriosis and control subjects. (a.) Volcano plot of RT^2 retinoic acid signaling pathway genes dysregulated between endometriosis and control subjects' ME-SFCs treated with vehicle for 6hrs. (b.) bar charts of retinoic acid pathway genes downregulated in endometriosis subjects' ME-SFCs. N=4 in all groups. *=p<0.05.

Differentially Regulated Genes in cAMP Stimulated Endometriosis Vs Control Samples

a.



b.

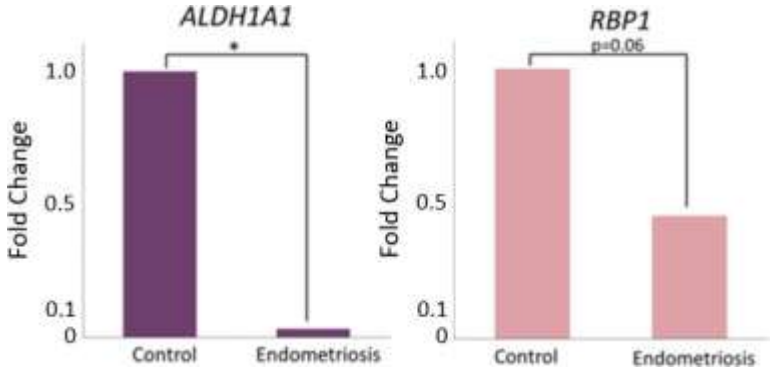


Figure 4. Differential expression of retinoic acid signaling pathway genes comparing ME-SFCs obtained from endometriosis and control subjects (±cAMP). (a.) Volcano plot of RT^2 retinoic acid signaling pathway genes dysregulated between endometriosis and control subjects' ME-SFCs stimulated with cAMP for 6hrs. (b.) bar charts of retinoic acid pathway genes downregulated in endometriosis subjects' ME-SFCs stimulated with cAMP for 6hrs. N=4 in all groups. *=$p<0.05$.

RBP1 gene expression is significantly reduced in endometriosis subjects' ME-SFCs

Due to the downregulation of *RBP1* mRNA expression observed in endometriosis lesions (Pierzchalski, et al. 2014a) and the slight decrease in *RBP1* mRNA expression by ME- SFCs of endometriosis subjects when compared to controls (n=4 per group), we investigated whether or not *RBP1* was down-regulated in ME-SFCs from endometriosis subjects when compared to ME-SFCs from controls using a larger sample size. *RBP1* mRNA expression was confirmed to be reduced in ME-SFCs of endometriosis subjects regardless treatment (vehicle vs. cAMP treatment (Figure 5). It is important to note that short-term (6hr) cAMP stimulation did not significantly alter the levels of *RBP1* mRNA expression (Figure 5). Since it has been shown that RBP1 is increased with in-vitro decidualization in endometrial stromal cells of eutopic endometrium from control women

undergoing hysterectomies, we decided to determine if *RBP1* mRNA expression could be increased at later time points during decidualization.

Discussion:

Dysregulation of the retinoic acid pathway has been recently identified (Cvetković, et al. 2003) as a potential contributing factor in the pathogenesis of endometriosis and the defects in decidualization associated with endometriosis (Pavone, et al. 2017; Pavone, et al. 2010; Pavone, et al. 2011; Pierzchalski, et al. 2014a; Taylor, et al. 2015). Reduced retinoic acid levels and proteins associated with the retinoic acid pathway have been found in endometriosis lesions when compared to eutopic endometrium (Pierzchalski, et al. 2014a). Pavone et al, have reported that when compared to eutopic endometrium from women without endometriosis, endometriosis lesions exhibit reduced expression of genes and proteins implicated in retinoic acid synthesis, including RBP1(Pavone, et al. 2017; Pavone, et al. 2010; Pavone, et al. 2011). By contrast, the endometriosis lesions were found to express increased levels of retinoic acid metabolizing enzymes and reduced levels of retinoic acid receptors when compared to the eutopic endometrium (Pavone, et al. 2017; Pavone, et al. 2010; Pavone, et al. 2011). Pierzchalski et al, also found reduced levels of RBP1 in endometriosis lesions, as well as reduced levels of retinoic acid and increased levels of retinol and retinol esters (Pierzchalski, et al. 2014a). In our study we also found reduced *RBP1* gene expression by ME-SFCs obtained from women with endometriosis (when compared to ME-SFCs from controls), as well as another retinoic acid synthesis enzyme, *ALDH1A1*.

Although we have observed a significant difference *ALDH1A1* expression by ME-SFCs from endometriosis subjects vs. control subjects treated with vehicle or cAMP for 6hrs, our study is limited due to our small sample sizes and should be repeated with ME-SFCs obtained from more subjects to confirm our findings. Additionally, the study is limited because the gene expression differences were found in cultured ME-SFCs rather than ME-SFCs directly isolated and immediately analzyed from menstrual effleunt. Culturing cells in vitro can lead to altered gene expression patterns, so not all in vitro findings will match in vivo data (Barragan, et al. 2016; Neumann, et al. 2010). In future studies ME- SFCSs should be isolated directly from menstrual effluent and compared to those propogated in culture, as well as compared between endometriosis and control subjects.

RBP1 is the major cellular retinol binding protein for the majority of tissues in the human body, since RBP2 expression is limited to the enterocytes of the intestine and RBP3 expression is limited to cardiac and skeletal muscle (Napoli 2017). In the endometrium RBP1 primarily acts to store retinol and make retinol more accessible to ALDH1A1 for conversion to retinal and then retinoic acid (Jiang, et al. 2018). RBP1 expression has been shown to change in endometrial stroma according to ovarian hormone fluctuations throughout the menstrual cycle (Orlandi, et al. 2004). The highest levels of RBP1 protein were found in the pre-decidua and decidua, similar to our findings and the findings of Pavone et al, showed who that RBP1 levels rise with in-vitro decidualization (Orlandi, et al. 2004; Pavone, et al. 2017). This led us to propose that RBP1 may play a role decidualization, either within its role in retinoic acid synthesis or in a yet to be discovered mechanism. However, our RBP1 knockdown model using ME-SFCs showed that knocking down RBP1 expression did not impair decidualization. Since RBP1 does not appear to play a strong role in decidualization, it seems unlikely that the reduction of RBP1 in endometriosis ME-SFCs contribute to endometriosis-associated infertility. This has led us to consider the potential role of RBP1 in the pathogenesis of endometriosis.

Future direction

Future studies should focus on whether, and how, reduced levels of RBP1 promote the development of endometriosis. Alterations in retinoic acid levels are likely to be the reason that loss of RBP1 promotes endometriosis and cancer growth. The loss of RBP1 in endometriosis lesions leads to lower levels of retinoic acid, which may lead to a pro- survival, pro-proliferative phenotype in endometrial cells destined to inhabit distant sites outside of the uterus. The idea that altering levels of proteins that bind to retinoic acid can affect cell functions is further supported by the work by Pavone et al, (Pavone, et al. 2010). Just as in endometrial and ovarian cancer, retinoic acid has been proposed as a treatment for endometriosis, for its tumor growth suppression and pro-differentiation effects (Cheng, et al. 2011; Tanabe, et al. 2008). Studies show that retinoic acid treatment can inhibit lesion growth in a mouse model of endometriosis (Wieser, et al. 2012). Retinoic acid stimulation of endometrial stromal cells has also been shown to alter the expression of genes related to proliferation (Yamagata, et al. 2015). Further studies are needed to confirm the ability of retinoic acids to prevent endometriosis lesion growth. Due to the teratogenic effects of retinoic acid, this would not be a reasonable treatment to propose for women with endometriosis who trying to become pregnant or become pregnant.
Targeting RBP1 rather than retinoic acid may confer the tumor suppressor effects of retinoic acid without the teratogenicity;

however, further studies need to be completed to confirm this.

Targeting RBP1 as a treatment would require a better understanding of its regulation and how it is downregulated in endometriosis lesions. RBP1 down-regulation in various cancers has also been found to be due to hypermethylation of CpG islands of RBP1 (Doldo, et al. 2015). The factors that cause hypermethylation of the RPB1 gene in cancer have not yet been identified; however, It is currently known that RBP1 expression is regulated by TGF-beta, all trans retinoic acid (ATRA), retinol, and ovarain steroid hormones (Doldo, et al. 2015). Alterations in TGF-β, ATRA, retinol, or estrogen and progesterone may contribute to the hypermethylation and down-regulation of RBP1; however, more work needs ot be done to make these connections. The epigenetics and regulation of RBP1 in stromal cells of endometriosis lesions, eutopic endometrium, and ME-SFCs needs to be further studied in order to identify ways to upregulate RBP1 to harness the anti-tumorigenic effects that might be useful fortreating endometriosis.

It is also unclear whether the reductions in proteins related to retinoic acid biosynthesis, metabolism, and signaling are acquired by endometriosis lesion as they develop in an inflammatory milieu at distant sites or if they are inherent to the cells that develop into endometriosis lesions (Taylor, et al. 2015). Our finding that *RPB1* expression is reduced in ME-SFCs from women with endomteriosis lends support to the hypothesis that RBP1 is inherently down-regulated in cells of the eutopic endometrium that may then form endometriosis lesions. This hypothesis should be explored in rodent models of endometriosis, where the capacity for lesions formation from endometrial tissue from mice that are RBP1 null can be compared to to those that express RBP1. However, these studies are limited by the fact that rodents do not menstruate or decidualize their endometrium.

The finding that RBP1 expression is altered in endometriosis ME-SFCs has opened up possibilities for diagnosis, understanding, and treatment of endometriosis. Further work needs to be done both in human samples and animal models to explore how RPB1 development contributes to endometriosis development.

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