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Altered Retinoid Signaling Compromises Decidualization in Human Endometriotic Stromal Cells

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ABSTRACT

Decidualization alters multiple molecular pathways in endometrium to permit successful embryo implantation. We have reported that paracrine factors, including retinoids, secreted from progesterone treated endometrial stromal cells act on nearby epithelial cells to induce the estradiol metabolizing enzyme HSD17B2. This same induction is not seen in endometriotic stromal cells. We have also shown significant differences in retinoid uptake, metabolism and action in endometriotic tissue and stromal cells compared to normal endometrium. Here we characterize retinoid signaling during decidualization in these cells. Endometrial and endometriotic were isolated, cultured and incubated and decidualized. Genes involved in retinoid metabolism and trafficking were examined using RT-PCR and western blotting. Prolactin, a decidualization marker, was also examined. We found that both endometrial and endometriotic stromal cells express all intracellular proteins involved in retinoid uptake and metabolism. Decidualization significantly reduced expression of the genes responsible for retinoid uptake and shuttling to the nucleus. However, expression of CRBP1, an intracellular carrier protein for retinol, increased, as did RBP4, a carrier protein for retinol in the blood which can function in a paracrine manner. Secreted RBP4 was detected in the media from decidualized endometrial cells but not from endometriotic cells. We believe that retinoid trafficking in endometrial stromal cells during decidualization may shift to favor paracrine rather than intacrine signaling, which may enhance signaling to the adjacent epithelium. There is blunting of this signaling in endometriotic cells. These alterations in retinoid signaling may help explain the decidualization defects, and deficient estradiol inactivation (via HSD17B2) seen in endometriosis.

Introduction

Endometriosis, an estrogen-dependent disorder, is often associated with pelvic pain and subfertility (Béliard, et al. 2004, Giudice and Kao 2004, Kennedy, et al. 2005, Nasu, et al. 2009, Wu, et al. 2008). It is estimated that 10% of all women and up to 30%-50% of symptomatic premenopausal women are affected by endometriosis, representing around 176 million women worldwide (Adamson, et al. 2010, Bulletti, et al. 2010, Bulun 2009, Giudice and Kao 2004, Nnoaham, et al. 2011, Stilley, et al. 2012). A recent study confirmed a high economic burden on endometriosis patients similar to that of other life-altering conditions such as diabetes and Crohn's disease (Simoens, et al. 2012).

Although endometriosis is a known cause of infertility or subfertility, there is debate as to whether it affects oocyte development, embryogenesis, or implantation. Studies investigating the effect of endometriosis on IVF outcomes are useful because they allow for the study of surrogate markers of reproductive success including peak estradiol levels, number of oocytes retrieved, fertilization, implantation, as well as pregnancy rates. By evaluating each component, it may be possible to determine the specific effects of endometriosis on reproductive outcomes. Barnhart *et al.* published a meta-analysis examining the effect of endometriosis on IVF outcomes (Barnhart, et al. 2002). The authors found that endometriosis negatively affected all aspects of IVF outcomes, including implantation rates. Furthermore, when comparing women with endometriosis to women with tubal factor infertility, all aspects of IVF outcome, including implantation rates, were lower (Barnhart, et al. 2002). This study also showed that disease severity also affected these outcomes, and that women with severe disease had a decrease in most aspects of IVF outcomes including implantation rates (Barnhart, et al. 2002).

Reduced implantation as well as altered receptivity in the endometrium may be caused by reduced capacity to decidualize. Decidualization is the response of maternal cells to progesterone in preparation for arrival of the blastocyst in early stages of pregnancy (Dunn, et al. 2003). During this process endometrial remodeling takes place by a secretory transformation of uterine glands, an influx of specialized uterine natural killer cells, and vascular remodeling (Gellersen, et al. 2007). Klemmt *et al.* found that endometriotic and endometrial stromal cells from women with endometriosis display reduced capacity for decidualization (Klemmt, et al. 2006). In comparison to stromal cells from healthy endometrium, stromal cells of ovarian endometriomas, peritoneal surface lesions, and deeply infiltrating lesions have a diminished ability to decidualize (Klemmt, et al. 2006).

Recent work has found reduced levels of all-trans retinoic acid (ATRA) in endometriotic lesions (Pierzchalski, et al. 2014). This is consistent with work from our lab which demonstrated that the retinoic signaling pathway is severely disrupted in endometriosis, leading to an overall decrease in genes involved in retinoid uptake and metabolism (Pavone, et al. 2011, Pavone, et al. 2010b). Specifically, we have shown significant differences in genes involved in retinoid uptake, metabolism, and action in endometriosis compared to normal endometrium. We have also demonstrated that paracrine factors, including retinoids, secreted from progesterone-treated endometrial stromal cells act on epithelium to alter steroid hormone response. These factors are absent in endometriotic stromal cells (Cheng, et al. 2007). We also have preliminary data showing that the expression of key genes in the retinoic acid signaling pathway are significantly lower in eutopic endometrium of baboons with experimentally induced endometriosis compared to controls during the window of implantation (Pavone, et al. 2010a). We hypothesize that decidualization alters the expression of integral genes in the retinoid signaling pathway. We hypothesize that even during decidualization, endometriotic cells would demonstrate a

molecular pattern consistent with decreased uptake and metabolism. We believe that these differences may ultimately contribute to the lower implantation rates seen clinically in endometriosis.

Materials and Methods

Tissue acquisition

All tissue used for this study was obtained using an Institutional Review Board approved protocol. Written consent was obtained from women suffering from benign indications before their surgeries. Eutopic endometrium was obtained from hysterectomies (n=17) and ectopic endometrium (endometriosis) was obtained from cyst walls of ovarian endometriomas (n=15) as has been previously described by our group (Pavone, et al. 2011). All the patients were pre-menopausal and were not on any hormonal therapy for at least 3 months prior to surgery. The phase of the menstrual cycle was determined by preoperative history as well as histological evaluation (all samples were in the follicular phase). The average age of the patients was 38±6 years.

Isolation and culture of primary stromal cells

Stromal cells were isolated using the protocol reported by Ryan *et al.* (Ryan, et al. 1994) with some slight modifications (Noble, et al. 1997, Ryan, et al. 1994) to maximize yield. Briefly, the tissues were minced and digested with pronase, deoxyribonuclease, collagenase (Sigma, St. Louis, MO) with constant shaking at 37ºC for 40 minutes, and if required, with hyaluronidase (Sigma) for another 20 additional minutes. Filtration through 70- and 20- µm sieves was used to separate stromal cells from epithelial cells. Stromal cells were then re-suspended in DMEM/F12 1:1 (Life Technology, Madison, WI) containing 10% Fetal Bovine Serum (FBS) with 100 µg/ml streptomycin and 100 IU/ml penicillin (Dyson, et al. 2014). A

humidified atmosphere at 5% CO_2 and 37ºC was used to grow the cells. Before the stromal cells were used for any experiments, they were passaged at least once.

In vitro decidualization

In vitro decidualization (IVD) protocol used was similar as previously reported (Kim, et al. 2007). Both human endometrial stromal cells (HESC) and endometriotic stromal cells (OSIS) were grown to 60-80% confluency, and then changed to IVD media consisting of phenol red-free DMEM/F12 media, 2% charcoal-dextran-stripped FBS (cs-FBS), and antibiotics as described above as well as 1 µM medroxyprogesterone acetate (MPA), 35nM 17β-estradiol (E_2) (both from Sigma), and 0.05mM 8-bromoadenosine 3',5'-cyclic monophosphate (cAMP, BIOLOG, Life Science Institute, distributed by Axxora, LLC, Farmingdale, NY). The control cells were maintained in cs-FBS media and treated with equal amounts of ethanol as a control (Dyson, et al. 2014).

Small interfering RNA (siRNA) knockdown

HESC and OSIS were grown to approximately 60-70% confluency. They were then transfected with either a non-targeting negative control siRNA (siCTL) (Dharmacon, Lafayette, CO), siRNA against Cellular retinoic acid binding protein 2 (*CRABP2*), or Fatty Acid Binding Protein 5 (*FABP5*) (both from Dharmacon) at a final concentration of 50nM using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA). After 12 hours of transfection, complete media was added for 24 hours. The cells were then subjected to IVD for a period of 4 to 6 days depending on the siRNAs used. IVD was performed in 2% cs-FBS. The cells were then harvested for RNA and proteins. Media was also collected for ELISA.

RNA extraction and quantitative real-time RT-PCR

Total RNA from HESC and OSIS was isolated using RNeasy columns (Quiagen, Valencia, CA) according to manufacturer's protocol. One microgram of RNA was used to generate cDNA using q-script cDNA SuperMix (Quanta Biosciences, Gaitherburg, MD). Real-time quantitative PCR was performed using Quant Studio 12K Flex and ABI Power Syber Green gene expression systems (Applied Biosystem, Foster City, CA). mRNA levels were quantified for Prolactin (*PRL*), *FABP5, CRABP2*, Retinol Binding protein 4 (*RBP4*), Cytochrome P450, Family 26, Subfamily A, Polypeptide 1 (*CYP26A1*), and *18S*. We have used *18S* as the control in many other papers (Pavone, et al. 2011, Pavone, et al. 2010b). Commercially available primers were used for all genes (Quiagen). Comparative threshold (CT) cycles method was used to perform relative quantification of mRNA species. For each sample, the gene CT value was normalized using the formula: ΔCT = CT gene of interest – CT18S (housekeeping gene). The following formula was used to determine relative expression level: ΔΔCT = ΔCT sample – ΔCT. This calibrator was used to plot the gene expression using the 2 – ΔΔCT formula. For IVD time course experiments, figures are normalized to mRNA expression on day 0 which we used as a control.

Preparation of protein

Whole cell lysates were prepared for protein quantification. Cells were washed with PBS homogenized using RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 50 nM Tris pH 7.6, 0.1% SDS, and 150nM NaCl) supplemented with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO). The lysates were centrifuged at 14000 x g for 10 minutes. Equal amounts of protein were resolved on NuPAGE Novex 4-12% Bis-Tris Gels (Life Technologies, Waltham, MA). Transfer and membrane blocking were performed as described (Dyson, et al. 2008). Incubation with primary antibodies was performed at 4ºC overnight (Cellular retinol binding protein 1 (CRBP1)) from Santa Cruz Biotechnology, Dallas, TX; Cytochrome P450; *CRABP2*, Family 26, Subfamily B, Polypeptide 1 (*CYP26B1*), from Sigma Aldrich (now Millipore Sigma), (St.

Louis, MO), FABP5 from Abcam (MA, USA); β-actin from Proteintech Group Inc (Il, USA) after which the membranes were washed and incubated with the appropriate HRP-conjugated secondary antibodies.

Epithelial cell culture and treatments

Endometrial epithelial Ishik awa cell lines (a gift from Dr. Masato Nishida Kasumigaura National Hospital, Tsuchiura, Ibaraki, Japan) were cultured in monolayers at 37ºC, 5% CO_2 incubator in DMEM and F12 (1:1) medium with 5% fetal bovine serum (FBS), 1% sodium pyruvate and 1% penicillin-streptomycin, hygromycin antibiotics solution (Life Technologies, Grand Island, NY). After the cell reached about 80% confluency, they were treated with RBP4 (Cayman Chemical, Ann Arbor, MI) at doses of 0.01µg/ml to 4µg/ml for 48 hours and real time RT PCR was done to test for HSD17B2. A time course was also done by treating Ishikawa cells with 1µg/ml serum RBP4 for 48 hours and testing the gene expression levels of HSD17B2 at 1, 12, 24, and 48 hours respectively.

ELISA

Media was collected from the cell culture plates and protein levels of Prolactin (Alpha Diagnostics, San Antonio, TX) and RBP4 (EMD Millipore, Billerica, MA) from endometrial and endometriotic cells were measured according to manufacturer's protocol. For IVD time course experiments, we normalized the protein values to the day 0 time point.

Statistical analysis

Non parametric testing (Kruskal-Wallis) was used to assess for statistical significance. A p-value <0.05 was considered statistically significant. Error bars represent standard error of the mean.

Results

IVD treatment increased PRL expression in HESC and to a much lesser extent in OSIS

As expected, *PRL* mRNA and protein secretion increased in both HESC and OSIS cells (Fig. 1), although to a far lesser extent in OSIS cells.

IVD treatment altered expression of genes in the retinoic acid signaling pathway

Figure 2 shows protein expression of *CRABP2, CRBP1* and *CYP26B1* when HESC and OSIS were subjected to a 14d IVD treatment. Interestingly, we observed that in HESC, there was an increase in *CRBP1* protein expression while *CRABP2* protein expression initially increased then decreased. In OSIS, expression of both of these proteins was much lower than in HESC. *FABP5* protein expression remained stable in both HESC and OSIS during IVD. *CYP26B1* expression is greater in OSIS compared to HESC.

Knockdown of CRABP2 in HESC cells decreases the ability of cells to decidualize

We previously showed that *CRABP2* is integral in determining cell fate in our model of endometrium and endometriosis (Pavone, et al. 2010b). Specifically, we showed that the *CRABP2*:*FABP5* ratio is different in endometrium and endometriosis, with a higher *CRABP2*:*FABP5* ratio in HESC and the reverse in OSIS. Further, we showed that when this ratio was altered, apoptosis and proliferation were affected. We also showed that the expression of *CRABP2* is decreased in OSIS compared to HESC. In order to examine if this decreased expression affected decidualization, *CRABP2* was knocked down in HESC for 48 hours, and cells were then decidualized for 4 days. As shown in Figure 3B, there was a significant decrease in the prolactin mRNA expression when *CRAPB2* was knocked down prior to IVD, indicating a decrease in the ability to decidualize. Results were confirmed by performing an ELISA for protein expression (Fig. 3C). This suggests that *CRABP2* expression is integral for proper decidualization to occur, and that a decreased expression of this gene prior to decidualization would affect the cell's ability to properly decidualize.

Knockdown of FABP5 in OSIS cells increases the ability of cells to decidualize

We have previously showed that *FABP5* is also important in determining cell fate in our model of endometrium and endometriosis. Specifically, we demonstrated that when *FABP5* was ablated in endometriosis, apoptotic markers increased and proliferation was decreased. Therefore, we explored what would happen when *FABP5* was knocked down in OSIS. We knocked down endogenous *FABP5* mRNA in OSIS for a period of 48 hours. After the knock down period, the cells were subjected to the IVD treatment for 4 days. As shown in Figure 4, there was a significant increase in *PRL* mRNA (Fig. 4B) and protein (Fig. 4C). This suggests that decreasing *FABP5* expression in endometriotic cells may increase the ability of the cells to decidualize. However, we do note that the level of prolactin secretion upon *FABP5* knockdown is minimal when compared to decidualizing eutopic cells. This, together with the results above, suggests that the *CRABP2*:*FABP5* ratio may affect a cell's ability to decidualize for both endometrial and endometriotic cells.

Decidualization Induces RBP4 Secretion

As shown in Figure 5, previous work has demonstrated that in normal endometrium, progesterone acts on stromal cells to induce secretion of paracrine factors that in turn act on neighboring epithelial cells to induce the expression of the enzyme 17β-hydroxysteroid dehydrogenase type 2 (HSD17B2) which catalyzed the conversion of estradiol to estrone (a much less potent estrogen) (Cheng, et al. 2007). In endometriosis, this enzyme is not induced with progesterone treatment because of a defect in paracrine signaling from stromal cells. One of these paracrine factors was thought to be retinoic acid. Retinol binding protein (RBP) is another paracrine factor acting as a specific carrier for retinol in the blood, and it has recently been shown that it can act as a signal to other cells (Yang, et al. 2005). Interestingly, we found that RBP4 was secreted during the decidualization of HESC cells, and a corresponding increase

was not found in OSIS cells (Fig. 5). We then incubated epithelial Ishikawa cells (same cells which had been used by Cheng *et al.*) with human serum RBP4 for 48 hours (time point established by Cheng *et al.*) and found that *HSD17B2* expression significantly increased (Fig. 5). We also found a dose-dependent induction (Fig. 5). This suggests that RBP4 may be an important paracrine factor secreted by endometrial stromal cells to induce expression of *HSD17B2*. RBP4 may be providing retinol that is metabolized into retinoic acid in epithelial cells. In addition, lack of its expression and secretion in endometriotic stromal cells would lead to a lack of *HSD17B2* expression and pathologically elevated local estradiol levels. RBP4 treatment also induced *CYP26A1* epithelial expression, another RA target gene (Supplemental Fig. 1).

Discussion

In this paper, we found that the genes in the retinoic acid signaling pathway are altered by decidualization (Fig. 6). Based on our prior work, we chose to focus on the actions of *CRABP2* and *FABP5* for this paper (Pavone, et al. 2011, Pavone, et al. 2010b). We found a consistent pattern of decreased expression of genes involved in retinoid uptake and action, and an increase in the expression of genes involved in retoinic acid elimination in endometriotic stromal cells subjected to decidualization. Taken together, this suggests that even during decidualization overall retinoid exposure in endometriotic cells is less than in normal endometrial cells. We also showed that expression of *CRABP2* and *FABP5* may be integral for proper decidualization to occur. We found that changing this ratio in endometrial cells decreased their ability to decidualize, while altering this ratio in endometriotic cells increased their ability to decidualize, as measured by prolactin expression and secretion. In addition, we found that decidualization induces the expression and secretion of RBP4 in endometrial stromal cells, and that RBP4 induces *HSD17B2* expression. This suggests that RBP4 may be the paracrine factor secreted by stromal cells that stimulates epithelial *HSD17B2* expression (Fig. 6).

Endometriosis is associated with pelvic pain and infertility. At least part of infertility is because of a decrease in the ability of an embryo to properly implant. Historically, endometrium has been investigated using an endometrial biopsy during the “window of implantation”, where normal histology was thought to be a reassuring finding. However, more and more studies are finding that although histologically normal, the endometrium of women during this window of implantation may be biochemically abnormal and that this may contribute to implantation failure. Lessey and collegues have demonstrated that even histologically normal endometrium from women with endometriosis lacks the $\alpha_V\beta_3$ inegrin which is a marker of uterine receptivity (Lessey, et al. 1992). Several other genes have been shown to be abberently expressed in the endometrium of women with endometriosis, inlcuding aromatase, HSD17B2, leukemia inhibitory factor, and progesterone recpetors (Giudice, et al. 2002, L Stewart 1994).

Human endometrium undergoes decidualization under the influence of progesterone (Aghajanova, et al. 2009). This can be done in vitro, as has been done in our study and many others (Fig. 1) (Aghajanova, et al. 2009, Pavone, et al. 2010b). Although progesterone is a key hormone in regulating the decidualization process, it has been demonstated at the molecular level that other pathways as well as other transcription factors are involved. As previously mentioned, impaired decidualization in both eutopic and ectopic endometirum has been described in the setting of endometriosis. Here we show that a number of proteins in the retinoic acid signaling cascade are altered by decidualization in both endometrial and endometriotic stromal cells (Fig. 2). We have previously shown that endometriotic cells express a molecular pattern consistent with decreased retinoid uptake, metabolism and action (Pavone, et al. 2011). Here we show that this is also true during decidualization. Namely, endometriotic stromal cells overall had a decrease in the expression of CRABP2 and CRBP1 with an increase in the expression of

CYP26B1, which allows for the elimination of retinoids from the cell. Pierzchalski *et al.* recently demonstrated defective ATRA biosynthesis in endometriotic lesions related to reduced expression of CRBP1. These studies suggest that defects in this gene resulted in abnormal retinoid biosynthesis, and that the ability of endometrial stromal cells to synthesize ATRA correlated well with their degree of decidualization (Pierzchalski, et al. 2014, Zheng, et al. 2000). Overall our prior studies, as well as these other findings, support the notion that during decidualization the retinoid signaling pathway is disrupted in endometriotic stromal cells. In addition, Sidell at al. found RA to be necessary for vascular endometrial growth factor (VEGF) secretion, which, along with endometrial angiogenesis, plays a critial role in successful implantation (Sidell, et al. 2010). After ovulation, VEGF production is largely limitied to epithelial cells This is consistent with our findings that RA metabolism is critical for proper decidualization to occur, and that RA metabolism in epithelial cells during decidulalization is crucial.

There is molecular dysregulation of the proliferative-to-secretory transition in women with endometriosis, suggesting resistance to progesterone action (Aghajanova, et al. 2009, Klemmt, et al. 2006, Yin, et al. 2012). Together with resistance to progesterone action, a deficiency in progesterone receptor (PR) has also been found in endometriotic tissues. It has been suggested that PR-deficiency may be responsibe for increased proliferation and resistance to apoptosis seen in endometriotic tissues (Bulun, et al. 2006). We have previously shown that *CRABP2* is regulated by PR, and that the resistance to apoptosis may, in part, be explained by decreased expression of *CRABP2* in endometriotic cells (Pavone, et al. 2010b). We have demonstrated that the ratio of *CRABP2*:*FABP5* is integral for determining cell fate in stromal cells from normal endometrium and endometriotic tissues. We showed that RA may promote differentiation and apopotisis in normal endometrium via *CRABP2*, while in endometriosis this pathway is shifted towards *FABP5* which mediated anti-apoptotic activity. In addition, we have previously shown that this is independent of nuclear recptor expression, namely RAR

296 and PPAR β/δ (Pavone, et al. 2010b). In addition, it has been reported that *CRABP2* expression was
297 correlated with the gain of ability to synthesize RA, and that this was important for the process of
298 embryo implantation (Bucco, et al. 1997, Bucco, et al. 1996). Here we show that *CRABP2* expression is
299 key for decidualization to occur properly. By decreasing *CRABP2* expression prior to exposing cells to IVD
300 media, prolactin expression and secretion was significantly decreased, suggesting a reduced ability to
301 decidualize (Fig. 3). Interestingly, we have also demonstrated that by decreasing *FABP5* expression prior
302 to inducing decidualization in endometriotic cells we were able to increase the expression and secetion
303 of prolactin, suggesting an induction of decidualization (Fig. 4), although to a much lesser extent than
304 what occurs in HESC.
305
306 HSD17B2, which is normally present in endometrial glandular cells but lacking in endometriotic cells,
307 inactivates estradiol. The lack of its presence in endometriotic lesions contributes to increased local
308 estradiol levels. In this paper and others, we have shown that the retinoic acid signaling pathway is
309 integral for proper HSD17B2 expression (Cheng, et al. 2007). Here we suggeset that RBP4 may be the
310 paracrine factor that transports retinol to the nearby epithelial cells. This retinol would be metabolized
311 to RA which would induce expression of *HSD17B2*, leading to estradiol inactivation (Fig. 5 and Fig. 6). We
312 have previously demonstarted that epithelial cells do express genes involved in retinol uptake and
313 metabolism (Mittal, et al. 2014). Absence of the expression and secretion of RPB4 from endometriotic
314 cells would contribute to increased local estradiol present in endometriotic cells.
315
316 RBP4 is the only specific transport protein for vitamin A, or retinol, in the circulation. It functions to
317 deliver retinol to tissues. Here we suggest that RBP4 may be the paracrine factor secreted by
318 decidualized endometrial stromal cells that activates *HSD17B2* to ultimately decrease local estradiol
319 levels. RBP4 has been shown to act as a paracrine factor in other studies (Ma, et al. 2016, Yang, et al.

2005). Specifically, it has been suggested that RBP4 may be a signal that contributes to the pathogenesis of type 2 diabetes (Yang, et al. 2005). Work by Berry *et al.* also supports the notion that RBP can act as a signaling molecule, and is able to delivery retinol to cells even in the absense of STRA6 (Berry, et al. 2013). Additionally, study found that fenretinide, a synthetic retinide, normalizes serum RBP4 levels and improves insulin resistance and glucose tolerance in mice with obesity inducted by a high-fat diet (Yang, et al. 2005). We have recently shown that treatment of endometriotic lesions with fenretinide induces apoptosis. In-vivo treatments were found to decrease lesion volume, and we hypothesized that an increase in retinol uptake may be the underlying mechanism of action (Pavone, et al. 2010a). Taken together with the results by a study by Yang *et al.*, this suggests that increasing retinol uptake may also increase retinol secretion and thus increase RBP4 secretion.

The major strength of this study is that all experiments were conducted using human tissues. One limitation is that we were unable to conduct the studies in eutopic endometrium from women with endometriosis. In our facility, most women with endometriosis undergoing uterine surgeries are also on medical management for endometriosis and pelvic pain, thus not ideal candidates for these types of studies. However, as mentioned in the introduction, we do have in-vivo data using a baboon model which supports our findings (Pavone, et al. 2010a). In addition, we were unable to measure actual retinoids in this study. We hope to be able to do this in the future.

In summary, we have demonstrated that gene expression in the retinoic acid signaling pathway is altered by decidualization. We suggest that impaired decidualization in endometriotic cells may be, in part, mediated by genes in the retinoic acid signaling cascade. We showed that expression of *CRABP2* and *FABP5* may regulate the cells' ability to properly decidualize. We have also demonstrated that RBP4 may be an important paracrine factor secreted by stromal cells to regulate epithelial expression of

HSD17B2. We believe that these molecular differences occuring in endometriotic stromal cells during decidualization could help explain the clinical decrease in implantation seen in women suffering from this disorder.

Declaration of interest: The authors have no conflict of interest to declare.

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Figure Legends

Figure 1: In vitro Decidualization (IVD) Induces Prolactin Gene Expression and Secretion in Endometrial Stromal cells and a Much Smaller induction in Endometriotic Stromal Cells: Human endometrial stromal cells showed an increase in (A) Prolactin (*PRL*) mRNA levels (shown as fold change). (B) Prolactin mRNA levels (shown as fold change). (C) Prolactin protein concentrations (ng/ml) when compared to endometriotic stromal cell. These experiments represent replications done in 14 patients.

Figure 2: Key Proteins Involved in Retinoid Trafficking in Endometrial and Endometriotic Stromal Cells During In-Vitro Decidualization: HESC and OSIS cells show key changes in proteins involved in the retinoic acid signaling pathway during 14 day in-vitro decidualization treatments. These experiments represent replications done in 10 different patients.

Figure 3: *CRABP2* Knockdown in Endometrial Stromal Cells Compromises Decidualization

(A) *CRABP2* mRNA expression (shown as fold change) is reduced even after 4 days in IVD cocktail. (B) *PRL* mRNA (shown as fold change) decreases significantly when *CRABP2* expression is ablated prior to IVD. (C) These results are confirmed by measuring *PRL* by ELISA (ng/ml). These experiments represent replications done in 6 different patients.

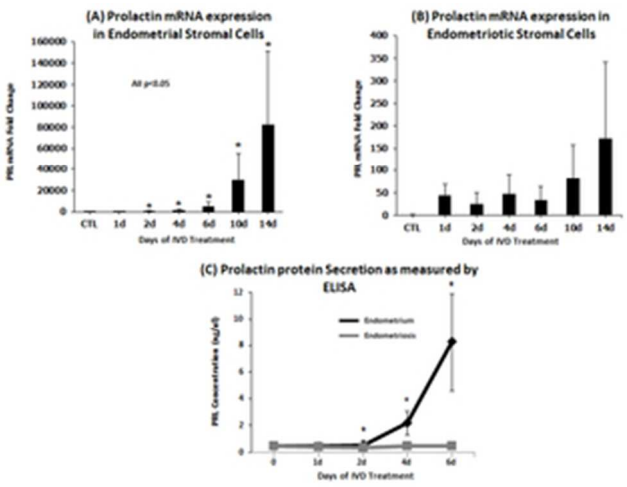
Figure 4: *FABP5* knockdown in Endometriotic cells Induces Decidualization:

(A) *FABP5* mRNA expression (shown as fold change) is reduced even after 4 days in IVD cocktail. (B) Prolactin mRNA (shown as fold change) increases significantly when *FABP5* expression is ablated prior to IVD. (C) These results are confirmed by measuring *PRL* by ELISA (ng/ml). These experiments represent replications done in 6 different patients.

Figure 5: Endometrial Stromal Cells Secrete RBP4 which Induces Epithelial HSD17B2 During Decidualization: (A) RBP4 mRNA is induced with IVD in endometriotic stromal cell, but not in endometriotic stromal cells. (B) We observed an increase in the RBP4 protein expression levels (ng/ml) in endometrial stromal cells during decidualization. (C) Treatment with RPB4 increased epithelial HSD17B2 mRNA expression (shown as fold change). (D) Dose response showing HSD17B2 expression after incubation with RBP4 (shown as fold change). These experiments represent replications done 6 times.

Figure 6: Schematic diagram of endometrial cells during decidualization (in the presence of E2 and P4): Genes in the retinoid pathway are altered during decidualization. During decidualization, *CRABP2*:*FABP5* ratios are integral for proper decidualization to occur. Decidualization also induces the expression and secretion of RBP4 in endometrial stromal cells, and that RBP4 induces HSD17B2 expression in nearby epithelial cells. This suggests that RBP4 may be the paracrine factor secreted by stromal cells that stimulates epithelial HSD17B2 expression. Impaired decidualization in endometriotic cells may be, in part, mediated by genes involved in the retinoic acid signaling cascade.

Supplemental Figure 1: Epithelial *CYP26A1* Expression in Induced by RBP4 Exposure: RBP4 is secreted by decidualizing cells and delivers retinol to epithelial (Ishikawa) cells. This experiment shows induction of another classic RA responsive gene, *CYP26A1*. This experiment represents replications done 3 times.



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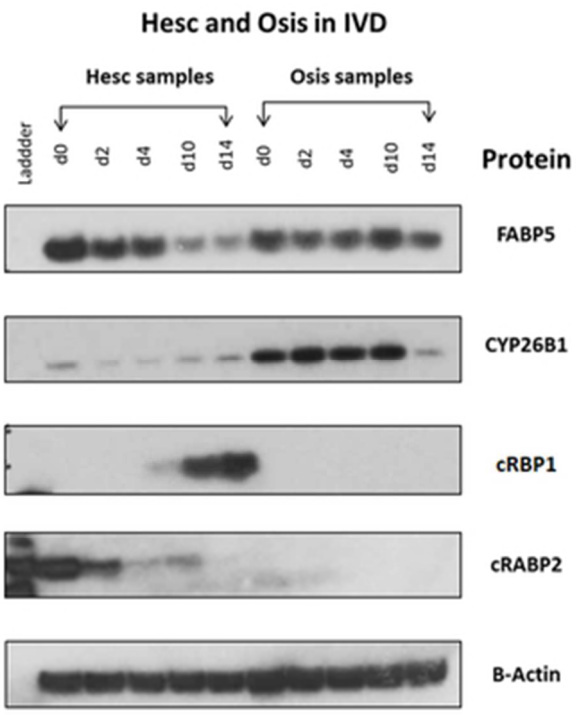

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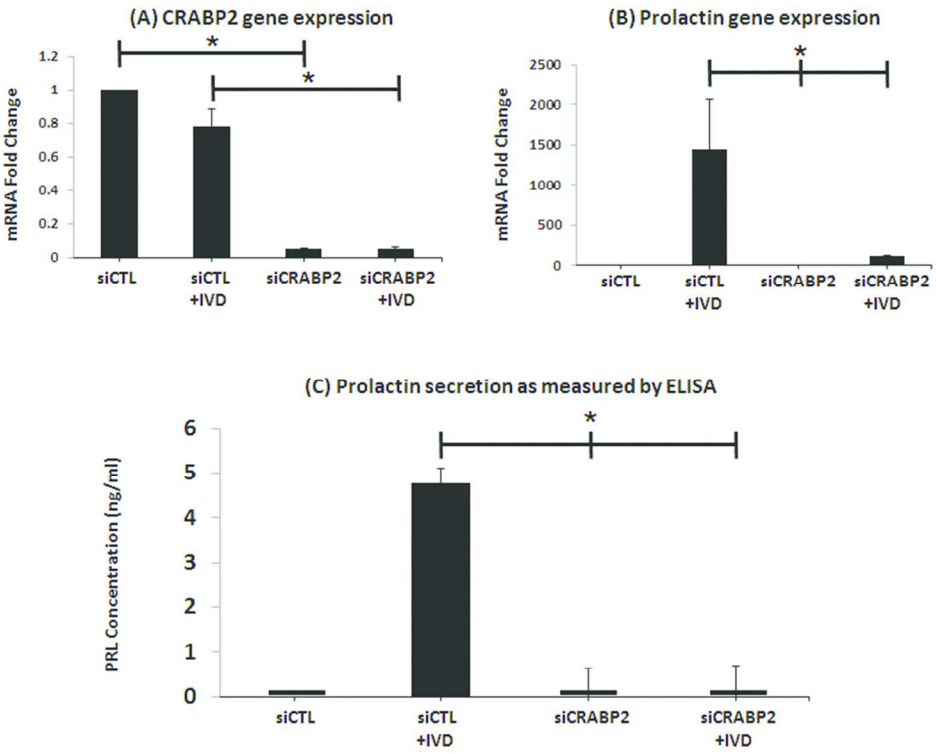

Figure 3: CRABP2 Knockdown in Endometrial Stromal Cells Compromises Decidualization: (A) CRABP2 mRNA expression (shown as fold change) is reduced even after 4 days in IVD cocktail. (B) PRL mRNA (shown as fold change) decreases significantly when CRABP2 expression is ablated prior to IVD. (C) These results are confirmed by measuring PRL by ELISA (ng/ml). These experiments represent replications done in 6 different patients.

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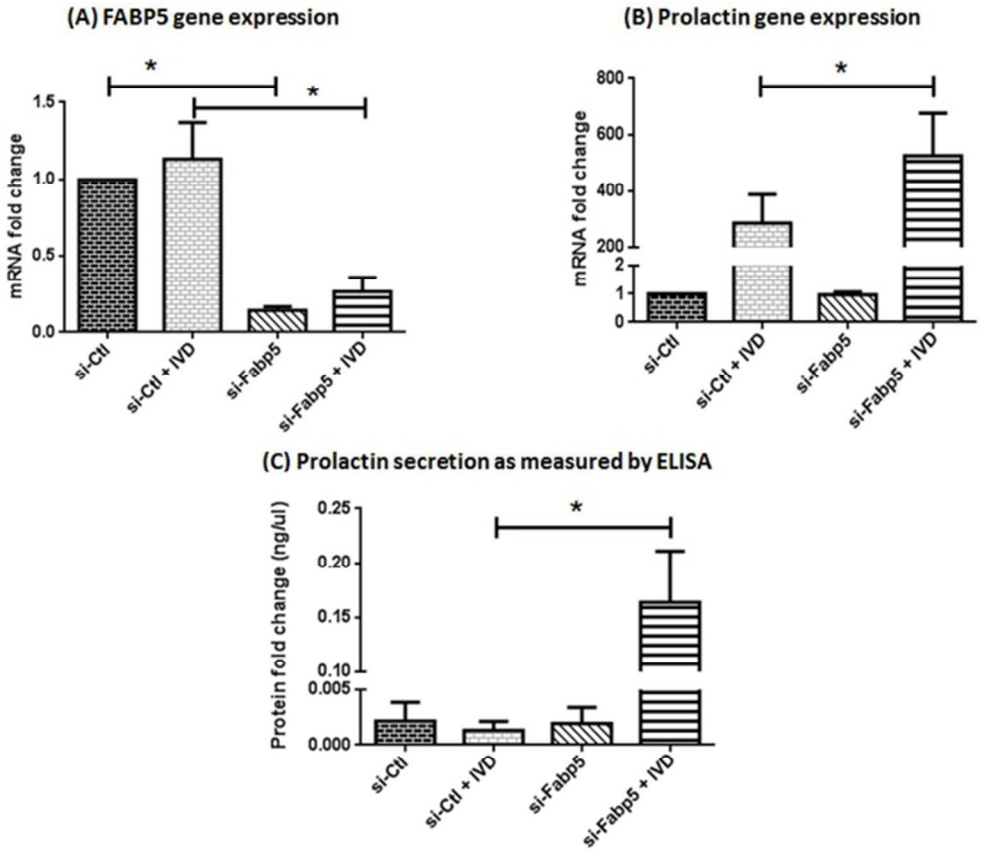

Figure 4: FABP5 knockdown in Endometriotic cells Induces Decidualization: (A) FABP5 mRNA expression (shown as fold change) is reduced even after 4 days in IVD cocktail. (B) Prolactin mRNA (shown as fold change) increases significantly when FABP5 expression is ablated prior to IVD. (C) These results are confirmed by measuring PRL by ELISA (ng/ml). These experiments represent replications done in 6 different patients.

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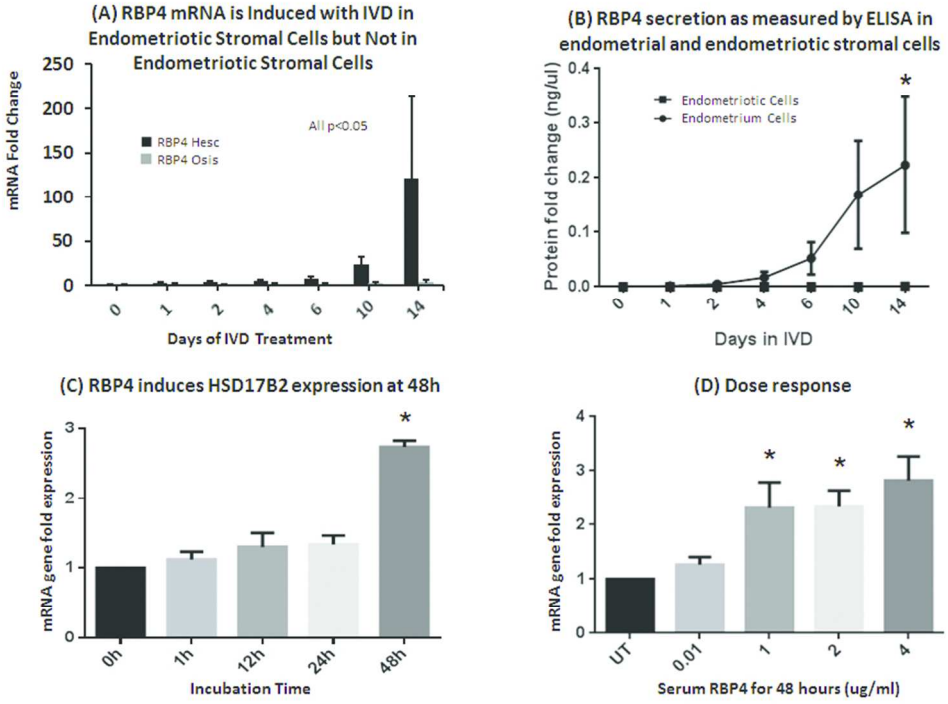

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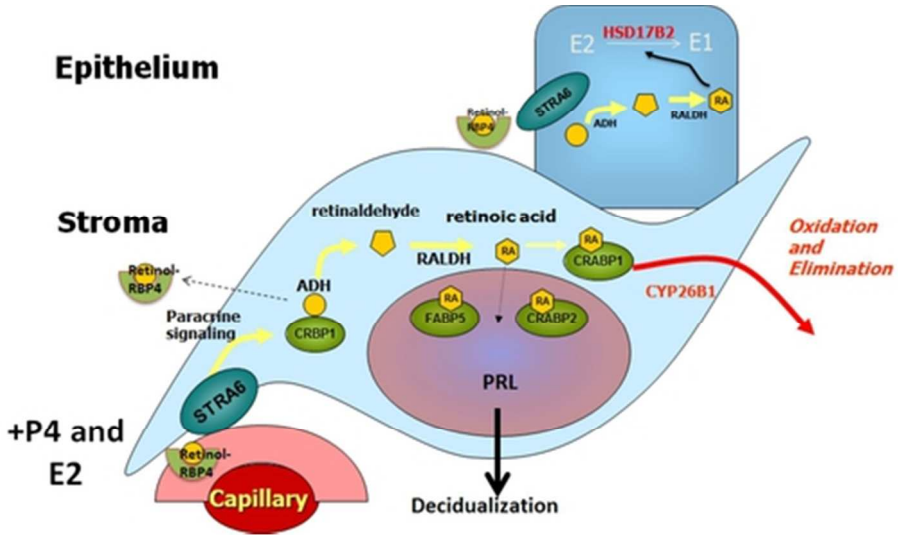

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