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Leveraging Genetics and Epigenetics to Understand Endometrial Function and Dysfunction

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Leveraging Genetics and Epigenetics to Understand Endometrial Function and Dysfunction

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Human Genetics

by

Katrina Nicole Leap

2023

ABSTRACT OF THE DISSERTATION

Leveraging Genetics and Epigenetics

to Understand Endometrial Function and Dysfunction

by

Katrina Nicole Leap

Doctor of Philosophy in Human Genetics

University of California, Los Angeles, 2023

Professor Julian Antonio Martinez, Chair

Uterine disorders are common and can be debilitating, but they are poorly understood. Endometriosis is the most common cause of secondary dysmenorrhea, yet existing treatments have substantial side effects and are not able to fully resolve pain for most patients. Many studies have shown that endometrial tissue from individuals with endometriosis has differential gene expression and DNA methylation patterns compared to endometrial tissue from controls. Data sharing efforts have made large amounts of genetic and epigenetic data available to researchers. However, it is technically challenging to compare datasets from different platforms and study designs, and these resources are underutilized. In this dissertation, I explore methods of combining DNA methylation and gene expression data to gain additional insights into endometrial disorders. First, I demonstrate that DNA methylation age can be used to understand ectopic endometriosis lesions. Then, I correlate DNA methylation age changes caused by hormonal treatment with differential gene expression in endometriosis. Given a candidate pathway, I then use datasets of infertility to determine whether these expression changes could

provide insight into clinical phenotypes. Finally, I explore whether clinical phenotypes of menstrual pain resistant to non-steroidal anti-inflammatory drugs (NSAIDs) can predict altered signaling of related pathways in menstrual-derived tissues. This work explores methods of utilizing the vast amounts of genetic and epigenetic data that have already been generated, while taking into consideration both the unique dynamic properties of uterine tissue and the aspects of uterine dysfunction that are most disruptive to quality of life.

The dissertation of Katrina Nicole Leap is approved

Steve Horvath

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University of California, Los Angeles

2023

This work is dedicated to menstruators everywhere, who deserve not to be in pain.

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Katie Leap, Iveta Yotova, Steve Horvath, & Julian A. Martinez-Agosto. Epigenetic age provides insight into tissue origin in endometriosis. *Scientific Reports,* **12**, 21281 (2022).

Justin W. Baldwin, **Katie Leap**, John T. Finn, & Jenny Smetzer. Bayesian state-space models reveal unobserved off-shore nocturnal migration from Motus data. *Ecological Modelling*, 386: 38-46, 2018.

CONFERENCE PROCEEDINGS

Katie Leap & Julian A. Martinez-Agosto. Decidualization-associated changes in epigenetic age are dysregulated in endometriosis and correlate with differences in DNA repair gene expression. Society for the Study of Reproduction Annual Meeting, 2022.

Katie Leap, Iveta Yotova, Steve Horvath, & Julian A. Martinez-Agosto. Leveraging epigenetics to understand ectopic tissue origin. World Congress on Endometriosis, 2021.

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Katie Leap, Julian A. Martinez-Agosto, & Steve Horvath. Using epigenetic age to investigate tissue origin in endometriosis. American Society for Human Genetics Annual Meeting, 2018.

Katie Leap, Steve Horvath, & Julian A. Martinez-Agosto. Genetic and epigenetic mechanisms of endometriosis. European Congress on Endometriosis, 2018.

Introduction

My dissertation research focuses on using genetic techniques to understand human uterine biology and addressing the technical challenges posed therein. These challenges include the dynamism of the uterus, considerations relating to the source of tissue studied, and ensuring that endometrial samples are relevant to phenotypes of concern to patients.

Uterine dynamics

The uterus is a reproductive organ best known for housing embryos, but it also facilitates the fertilization of eggs[1], coordinates the implantation of the blastocyst, provides the interface between the fetal placenta and the maternal decidua, and ultimately expels both fetus and placenta from the body[2]. These functions require the uterus to be dynamic: to change over time and to respond to stimuli.

There are three layers in the uterus: 1) perimetrium: the outer layer, a thin protective layer; 2) myometrium: the middle layer, the thickest layer, that consists of smooth muscle; and 3) endometrium: the innermost layer, which grows and sheds in the menstrual cycle. Endometrium is a structured tissue that owes its shape to both stromal cells and epithelial glandular cells, as well as arterial support[3]. Uterine function is coordinated between the endometrium and the myometrium; while the endometrium grows and sheds during the menstrual cycle, the myometrium grows and contracts to accommodate pregnancy[4].

Across the lifespan, the uterus changes in ways that are more complex than just growing in size. The uterus develops early in embryogenesis and fetal endometrium can be seen as early as 4 months into development[5]. By the late gestational period, the fetal epithelial cells parallel those of sexually mature women, including mature glands. Infant endometrium does not

resemble adult endometrium exactly, but the cells present are as hormonally responsive as they would be in an adult.[6]

Because the uterus is responsive to hormonal stimuli, the fetal uterus responds to circulating maternal hormones and the size of the uterus is largest toward the end of the gestational period. Further evidence suggests that progesterone receptivity may induce an inflammatory reaction capable of decidualizing endometrial stromal cells, regardless of ovarian activity[7]. After birth and the resulting loss of hormonal input, uterine volume decreases from a median of 3.4 mL to about 1 mL and remains static until puberty, even as the rest of the body grows[8]. The pre-pubertal uterus is tubular, and the cervix is longer than the body of the uterus[9]. Breast growth is the hallmark of female puberty, but uterine growth accelerates before the growth of breast tissue[10]. This growth is unrelated to height, weight, or age, and the uterine body grows to double the size of the cervix, reaching its maximal, nulliparous size around the age of 20[11].

The next major stage for the uterus is pregnancy. Pregnancy causes the uterus to grow to accommodate the growing fetus and the process by which the uterus returns to its former size is called involution. The endometrial lining of the puerperal uterus is shed completely in the form of considerable postpartum bleeding and the myometrium contracts to return the uterus to a smaller size[4]. The final dynamic of uterine size is post-menopause when the endometrial layer thins due to the loss of circulating hormones, rendering the body of the uterus smaller than the cervix[9].

In addition to changing across the lifespan, the uterus has the ability to remodel drastically during the reproductive years. The inner lining of the uterus, the endometrium, has two layers: the functionalis layer that grows and sheds, and the basalis layer, which does not.

The menstrual cycle describes how the endometrium responds to the hormonal outputs of the ovarian cycle. During the follicular phase of the ovary, the endometrium responds to estrogen by thickening, termed the proliferative phase. After ovulation, the corpus luteum in the ovary begins to produce progesterone that signals the endometrium to enter the secretory phase. In the absence of fertilization, the corpus luteum will atrophy and the loss of progesterone will initiate menstruation, the shedding of the functionalis layer. The endometrium does not cycle during pregnancy or while a person with a uterus is taking hormonal contraceptives, which is a pseudo-pregnancy[12].

The lack of menstrual bleeding is called amenorrhea and it is expected during pregnancy, lactation, pre-puberty, and post-menopause. After the onset of menses (menarche), amenorrhea can be caused by anovulation, which is common in puberty, or by hormonal disturbances[13]. Excessive menstrual bleeding is referred to as heavy menstrual bleeding, formerly called menorrhagia[14], while pain with menstruation is known as dysmenorrhea[15].

Sampling the uterus

Menstruation is not common in mammals and is only observed in primates, elephant shrews, some species of bat, and one species of mouse, the spiny mouse[16]. Because of this, research on the function and dysfunction of the endometrium is typically done in humans. Endometrial samples can be obtained by endometrial biopsy, which is a cylindrical section of the endometrium. Prior to processing, the menstrual phase is determined histologically[17]. Samples can be processed and analyzed as intact tissue, which will consist of several cell types, or as isolated cell populations that can be processed or cultured. Cell isolation techniques fall under microdissection or collagenase treatment and filtration. Microdissection can be performed manually or with the assistance of a laser and the isolated cell populations can be processed for

DNA, RNA, or proteins to be studied[18]. Collagenase treatment will break the connections between cells so they can be separated. Epithelial cells and stromal cells are of different sizes and can be isolated by suspending the cells in a liquid and passing the suspension through a mesh filter that will capture the desired cell type[19]. This form of isolation allows for the cells to be grown in culture, which offers the ability to perform experiments with the cells or grow a larger number if the input material is small.

Another source of endometrial tissue comes from hysterectomy, the removal of the uterus. These samples allow glands to be extracted in their entirety, rather than retrieving a cross section. Cells can be isolated and processed the same as endometrial biopsies, but more is known about the spatial characteristics of the cells. A downside to sourcing endometrium from hysterectomies is that they are typically performed in older patients, after desired family size is achieved, or in patients with uterine pathologies[20].

When studying endometrial tissue, it is important to know when in the menstrual cycle the sample was collected, the composition of cell types in the sample, and the age of the donor (Figure 1-1).

Uterine dysfunction

Uterine dysfunction usually manifests in the form of diseases of the endometrium, including endometriosis, adenomyosis, uterine fibroids, infertility, and endometrial cancer. Unlike endometrial cancer, most of these conditions are understudied and poorly understood. This is likely due to a combination of complex biological processes and a lack of unique approaches to identify their etiologies.

Endometriosis is a reproductive disorder wherein endometrial tissue is found outside of the uterus and is staged according to the size and extent of ectopic lesions. However, while

clinical staging of the disease is extensively correlated with its effect on fertility, it does not incorporate the major symptom of the disease: amount of pain experienced[21]. Individuals with endometriosis-associated pain have a lower quality of life and are less likely to be employed than those with asymptomatic endometriosis and healthy controls[22,23]. Menstrual pain is thought to be a process of uterine contractions that are initiated by prostaglandins and popular over-the-counter (OTC) painkillers work by inhibiting the enzyme that produces prostaglandins, cyclooxygenase (COX)[24]. Many people with endometriosis do not experience adequate pain relief from OTC painkillers for their pelvic pain, and pain remains one of the main challenges in managing and treating this condition[25].

An intuitive model of the disease might posit that pain severity and location would correspond with extent and location of ectopic growths; however, patients with extensive ectopic growths can present with no pain and not be diagnosed until they are unable to conceive, while those with debilitating, localized pain can have small growths not specific to the location of their pain[26]. Furthermore, people with endometriosis experience more severe gastrointestinal symptoms than healthy people and these symptoms are not affected by hormonal treatment[27], suggesting that some symptoms of endometriosis may not be under hormonal control. While endometriosis is known to be genetic, with twin studies describing up to 50% heritability, it does not follow Mendelian patterns and candidate gene and linkage analysis studies have not identified any single gene associated with endometriosis[28]. Modeling endometriosis as a complex trait instead, meta-analysis of genome-wide association studies (GWAS) has identified 19 single nucleotide polymorphisms (SNPs) that together explain 5% of the variance in endometriosis[29]. Research into splice variants in endometriosis have found differential expression of splice variants of KRAS[30] and CD44[31] in the endometrium during

menstruation and of estrogen receptor alpha in the endometrium during the proliferative phase that correlated with severity of menstrual pain[32].

In this dissertation, I will explore methods of utilizing the vast amounts of genetic and epigenetic data that have already been generated, while taking into consideration both the unique dynamic properties of uterine tissue and the aspects of uterine dysfunction that are most disruptive to quality of life.

Figure 1-1

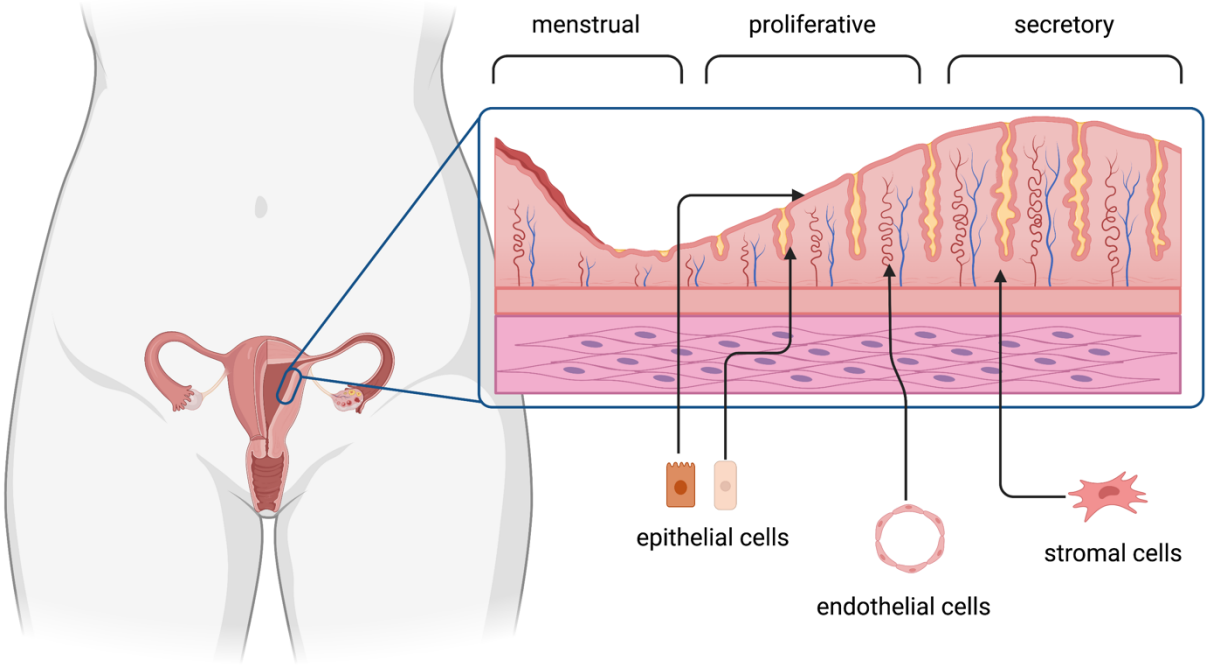

Figure 1-1: Endometrial phases and cell types

The uterus is a small organ situated low in the pelvis. The endometrium is a thin layer over the much thicker myometrium layer of smooth muscle. Endometrium has different thicknesses depending on the current hormonal situation: it is thinnest right after menstruation or post-menopause and thickest during the secretory phase. Hormonal contraceptive use renders the endometrium thinner than natural cycling would. The three major cell types are shown: epithelial cells which line the glands and uterine cavity, endothelial cells which constitute the blood vessels, and stromal cells which fill the space between. Endometrial biopsies consist of these cells, as well as blood and immune cells, and their thickness and cell type composition will be dependent on the menstrual phase. Created with BioRender.com

Chapter 1 : Epigenetic age provides insight into tissue origin in endometriosis

scientific reports

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Epigenetic age provides insight into tissue origin in endometriosis

Katie Leap[1], Iveta Yotova[2], Steve Horvath[1,3,4] & Julian A. Martinez-Agosto[1,5]✉

Endometriosis is a common reproductive disease with a heterogeneous presentation. Classification attempts have thus far not offered insight into its cause or its symptoms. Endometriosis may result from the migration of shed endometrium to the peritoneal cavity. However, there are cases reported in girls without uteruses and men. While a non-retrograde menstruation origin of ectopic tissue is certain in these cases, we explored the use of DNA methylation age (DNAm age) to distinguish between retrograde and non-retrograde tissue origin in endometriosis. Using publicly available DNA methylation data and Horvath's pan-tissue epigenetic clock, we compared DNAm age and epigenetic age acceleration (EAA) of ectopic lesions to eutopic endometrium of diseased and control endometrium. We examined EAA in cancer metastasis and teratomas to control for migration and developmental origin. Disease status does not change DNAm age of eutopic endometrium, but the effect of ectopic status was profound: −16.88 years ($p = 4.82 \times 10^{-7}$). There were no differences between EAA of primary/metastatic tumor paired samples, suggesting that the observed effect is not due to tissue migration or ectopic location. Immature or mature teratoma compartments decreased DNAm age by 9.44 and 7.40 years respectively, suggesting that developmental state correlates with DNAm age. Ectopic endometriotic tissue exhibits decelerated DNAm age, similar to that observed in teratomas composed of multipotent tissue, but distinct from eutopic tissue. The migration process does not change DNAm age and eutopic endometrium is concordant with chronological age regardless of disease status. We conclude that DNAm age of ectopic lesions suggests a distinct developmental origin for a subset of lesions. This finding may assist in classifying endometriosis into distinct subtypes that may be clinically relevant.

Endometriosis is a common reproductive disease that affects up to 10% of women defined by the presence of endometrium-like tissue in ectopic locations[1], but the presence or size of ectopic endometriotic lesions does not predict fertility or the type or severity of pain experienced[2]. Attempts to classify the disease according to surgical, clinical or molecular features have thus far not offered any insight into what causes the disease or its symptoms[3]. We therefore explored the use of DNA methylation age to predict tissue origin with the hope that this approach will enable better understanding of the disease and tailored treatment for affected women.

DNA methylation is an important part of gene regulation and is essential to development. During embryogenesis, the zygote undergoes dramatic DNA methylation changes, where many marks are erased and reestablished. DNA methylation continues to change throughout the lifespan of an individual in a predictable way such that the age of a person can be reliably predicted from the DNA methylation of a donated tissue[4]. One such method is Horvath's pan tissue clock, which has as its input methylation values and as its output a predicted age in years[5]. Although DNA methylation patterns are variable between tissue types, the pan tissue clock applies to all nucleated cells. Most tissues have roughly the same age according to the pan tissue clock with the exception of female breast tissue and the cerebellum[5–7]. Furthermore, it predicts an embryological age, i.e. close to 0 years, for induced pluripotent stem cells, indicating that the biological process underlying the clock may be related to tissue status along the differentiation-senescence timeline[4]. We hypothesized that this property of the pan tissue clock (young age estimate for stem cells) might be beneficial in the context of establishing tissue origin, particularly for conditions like endometriosis in which this question remains a challenge.

Endometriosis is thought to be caused by the migration of shed endometrium to the peritoneal cavity through retrograde menstruation. Recent studies have demonstrated this disease mechanism by implicating mutations originating within the glands of the endometrium in the pathogenesis of endometriosis-associated ovarian

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cancer[8,9]. However, there are reported cases of endometriosis in girls who have not menstruated or who do not have uteruses, as well as in fetuses and in men[10–12]. While a non-retrograde menstruation origin of ectopic tissue is certain in these cases, we propose that the pan-tissue clock can distinguish between uterine and non-uterine etiologies in the commonly observed ambiguous cases. Here we demonstrate that ectopic endometriotic tissue exhibits a younger than expected (i.e. decelerated) DNAm age, similar to that observed in teratomas composed of multipotent tissue. The process of migration does not change DNAm age, and eutopic endometrium is concordant with chronological age regardless of disease status. Our findings suggest that DNAm age deceleration of ectopic lesion derived stromal cells may indicate that cells within the lesions have either aged more slowly and retained some degree of developmental immaturity or experienced a DNAm age deceleration as part of the disease process.

Materials and methods

Data selection and study population. All data used in this analysis was publicly available from the Gene Expression Omnibus (GEO); accession numbers, sample sizes, and platform information can be found in Supplemental Table S1. We used every methylation dataset available on GEO found with the search term "endometriosis" that used endometrial tissue or cells (Supplemental Fig. S1). Given how small these datasets of endometriosis tissues are, we had neither the power nor the variation necessary to study the entire methylome. Instead, we distilled the methylome into one biologically relevant metric and used larger datasets with known cell migration or developmental properties to identify how the metric changes under these conditions. We chose datasets for the metastatic cancer analysis based on the following inclusion criteria: presence of tissue from both primary and metastatic tumors, availability of age information, and platform compatibility. To investigate tissues of a developmental origin, we used a dataset of intracranial germ cell tumors, which are classified into five different types: germinomas, embryonal carcinomas, teratomas, yolk sac tumors, and choriocarcinomas[13]. We focused on teratomas because of their developmental origin and their low likelihood of being malignant[14]. Patient characteristics of control and patient groups have been previously described[13].

Horvath's pan-tissue clock. We chose to use Horvath's pan-tissue clock for the following reasons: (1) it applies to all nucleated cells and tissues, (2) it lends itself to comparing the ages of different tissues[5,6], (3) it leads to a very low age estimate in the case of stem cell types, and (4) it is validated in both 27 k and 450 k Illumina platforms[5]. Full details on DNA methylation age and epigenetic age acceleration calculated using Horvath's pan-tissue clock are described elsewhere[5] and the software for calculating these metrics is hosted online (https://horvath.genetics.ucla.edu/html/dnamage/). Briefly, DNA methylation age represents the predicted age of a tissue in years. If the clock is performing well, there will be a strong positive correlation between predicted age and actual age; we expect this correlation in healthy tissues, but relax the assumption for diseased tissues. Because age cannot be negative and is thus bounded at 0, there is more variability in predicted age as actual age increases. This means that the same difference between predicted and actual age in a child is more significant than in an older person. Therefore, we do not use raw differences to represent age acceleration (or deceleration), and instead use the residual from the model predicting DNAm age from actual age, which we call epigenetic age acceleration and can be positive or negative. The sensitivity of the clock is 3.6 years as determined by the median absolute difference between DNAm age and chronological age in the testing data used to develop the method.

Statistical analyses. Before conducting any analyses, we confirmed correlation of DNAm age with chronological age using Pearson's correlation coefficient as well as graphically (Fig. 1A).

We used epigenetic age acceleration (EAA) initially to compare different tissue types graphically with boxplots. However, we chose to use age as a covariate and predict DNAm age using ordinary least squares regression because age was missing from some of our samples and EAA cannot be calculated if age is missing. We fit three different models: (a) an endometriosis model with the following covariates: age, diagnosis of endometriosis (binary), menstrual phase (binary: 0 for secretory, 1 for proliferative), ectopic status (binary), and stromal status (binary); (b) a metastatic cancer model with the following covariates: age and metastatic status via three binary indicator variables: primary tumor with metastases, primary tumor without metastases, and metastatic tumor; and (c) a teratoma model with the following covariates: age, tumor content of the sample (percentage) and non-mutually exclusive binary indicator variables for embryonic carcinoma, germinoma, immature teratoma, mature teratoma, seminoma and yolk sac tumor. Age estimates for each model can be seen in Fig. 1B. The Akaike Information Criterion (AIC) was used to select the best model. Significance for each coefficient was determined using a t-test and confidence intervals were bootstrapped. Paired analyses of DNAm age were conducted using paired t-tests. Effect sizes smaller than 3.6 years were not considered significant because of the sensitivity of the epigenetic clock. We did not correct for multiple testing because we used one model for each analysis, but we have reported raw p values.

Missing data. Out of 685 samples in the metastatic cancer dataset, 88 were missing metastatic status, most frequently because stage of the cancer was available but no information about the metastatic status of the particular tumor sample was available. The difference in DNAm age between the tissues with known metastatic status and unknown metastatic status was 2.4 years ($p = 0.27$). As the DNAm age was not significantly different and was within the margin of error of the clock (3.6 years), we considered these to be missing at random and excluded them from our analyses.

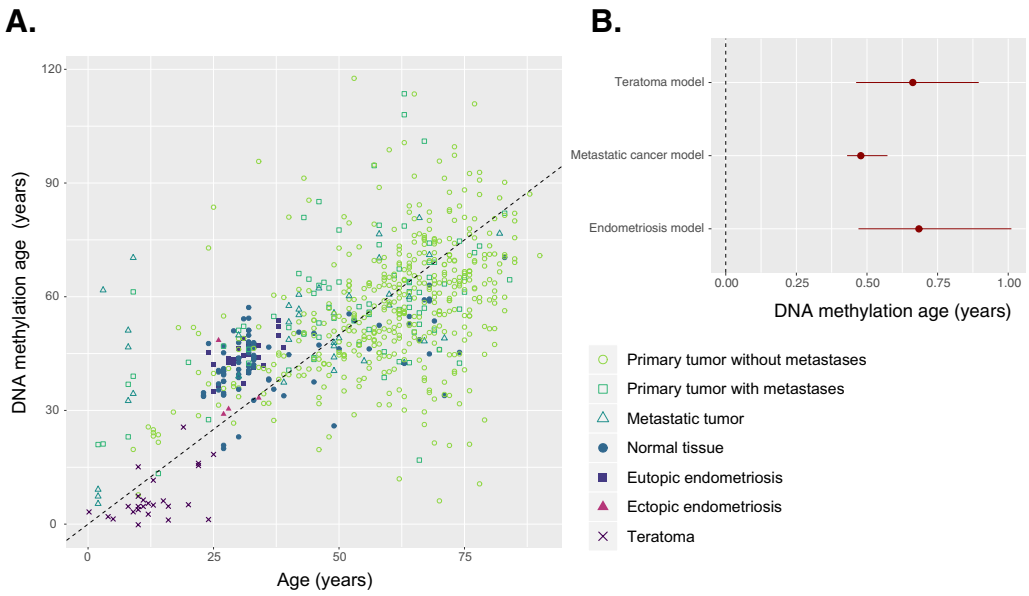

Figure 1. (**A**) Correlation between chronological age and predicted DNA methylation age. Dashed line indicates the identity line where perfectly correlated points would be found. Open shapes are cancerous tissues; filled shapes are non-cancerous. (**B**) Age parameters estimated from each of the three multivariate models; point estimate and bootstrapped 95% confidence interval are shown. A value of 0 would indicate that age does not affect DNAm age; a value of 1 would indicate that for every 1 year increase in age, DNAm age increases by 1 year.

Results

DNA methylation age of eutopic endometrium is not affected by endometriosis status but menstrual phase has a small effect. We first tested whether the DNAm age of eutopic endometrium of women with and without endometriosis is concordant with chronological age using Pearson's r and found a positive correlation of $r = 0.6$ ($p = 7 \times 10^{-6}$), which is consistent with previous findings in uterine endometrium[5]. A recent study found a correlation of 0.8 between DNAm age and chronological age in uterine endometrium when all of the samples came from the same menstrual time point (LH+7, or 7 days after the surge in luteinizing hormone)[15], but our correlation was unchanged if we stratified by menstrual phase (LH+8), or by endometriosis status. We next tested the effect of menstrual phase on DNAm age because menstrual phase is known to affect DNA methylation[16–18]. Each of the healthy controls donated two samples, one during the early secretory phase and the second during mid-secretory phase of the same menstrual period[19]. These two time periods are just before and just after the differentiation of the stromal cells to decidual cells at the beginning of the receptive phase of the endometrium[20]. If the clock is tracking differentiation, we might expect an older age after this change. The average difference in DNAm age between these paired samples was 2 years ($p = 0.07$), which is within the sensitivity of the clock, and the correlation between the two DNAm ages was 0.7 ($p = 0.001$), a stronger correlation than with chronological age (Fig. 2). However, when samples are compared in aggregate, this small change is no longer significant (Fig. 2A). This implies that menstrual phase has a limited effect on DNAm age, both in timing and magnitude. Finally, we tested the repeatability of DNAm age: the mid-secretory phase samples each had a technical replicate and the correlation between the DNAm age of these paired replicates was incredibly strong at $r = 0.99$ ($p = 4 \times 10^{-15}$). Given that DNAm age is correlated with age in the eutopic endometrium, menstrual phase has a small effect, and the predicted age is repeatable, we tested whether endometriosis would affect DNAm age of the eutopic endometrium. In our full linear regression model, we predicted DNAm age given chronological age, ectopic status, endometriosis status, menstrual phase, and stromal cell status. The effect of disease status was not significant (0.98 years, $p = 0.344$) indicating that disease status does not change DNAm age in the eutopic endometrium (Figs. 2A, 3B).

Ectopic endometriotic tissue is epigenetically younger than eutopic endometrium. We next compared the DNAm age of ectopic endometriotic lesions with that of eutopic tissue. Ectopic tissue was substantially younger than expected, exhibiting a profound age deceleration: −16.88 years ($p = 4.82 \times 10^{-7}$, Fig. 2B). As ectopic lesions are often located on the ovary, as were all of the samples used in this analysis, we could not completely exclude adjacent tissue contamination, which might consist of ovarian epithelium, stroma, or germ cells. However, it is unlikely that the effect of ectopic status on DNAm age reflects adjacent ovarian tissue contamination for the following reasons. First, by construction, the pan-tissue clock is quite robust to differences in cell composition[5]. Second, the ectopic endometriosis data came from primary stromal cells isolated from surgical

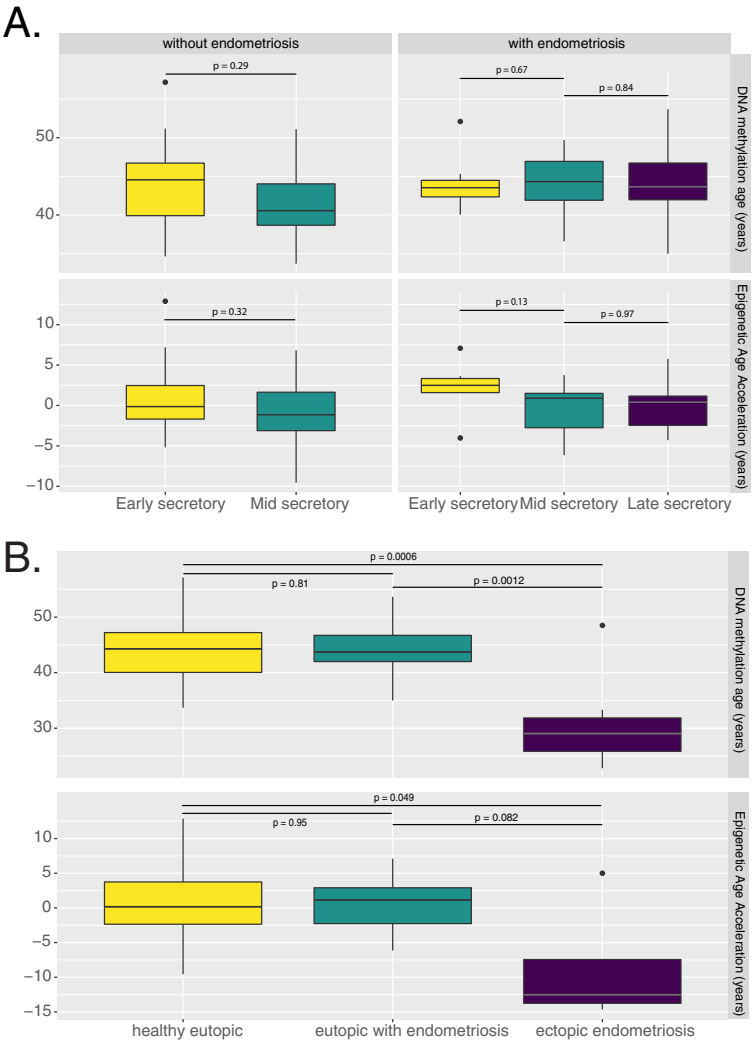

Figure 2. (**A**) Menstrual phase and disease status do not have a significant effect on DNA methylation age (top) or Epigenetic Age Acceleration (bottom). All samples are of eutopic tissue, coming from 26 women with endometriosis (early secretory: n = 7, mid secretory: n = 10, late secretory: n = 9) and 17 women without endometriosis who each donated samples at early- and mid-secretory phases of the same menstrual cycle. *P* values for paired comparisons using the Wilcoxon rank sum test are included. (**B**) Ectopic endometriotic lesions are significantly younger than eutopic samples as seen in the boxplot; midline is the median, box edges represent the interquartile range. Healthy eutopic: n = 42; eutopic with endometriosis: n = 29; ectopic endometriosis: n = 7.

tissue samples thereby representing a pure population of cells, grown ex vivo for not more than three passages[21]. Third, we trained a logistic regression classifier on the full methylome to predict whether a tissue was ovarian or not and determined that the ectopic lesions were not likely to be ovarian. Overall, it is unlikely that the substantially younger DNA methylation age of ectopic tissue reflects artifactual contamination with ovarian tissue.

Stromal cell identity does not explain age deceleration. Because all of the ectopic samples consisted of cultured stromal cells, we considered whether cultured stromal cells might exhibit age deceleration. Mesenchymal stromal cells derived from human bone marrow have previously been shown to have a DNAm age that is correlated with chronological age and therefore lacking any age deceleration. When these cultured stromal cells were reprogrammed to become induced pluripotent stem cells (iPSCs), their DNAm age decreased to zero, but differentiation back into mesenchymal stromal cells did not increase their DNAm age[22]. This supports our finding in the endometrium that differentiation does not advance Horvath's pan-tissue clock and does not give us reason to suspect that stromal cell identity could be driving ectopic epigenetic age deceleration. We examined eutopic stromal cells to see if they exhibit any epigenetic age acceleration or deceleration as compared to the whole tissue (Fig. 3A). Our results found that if we were to hold all other variables constant, stromal cells would

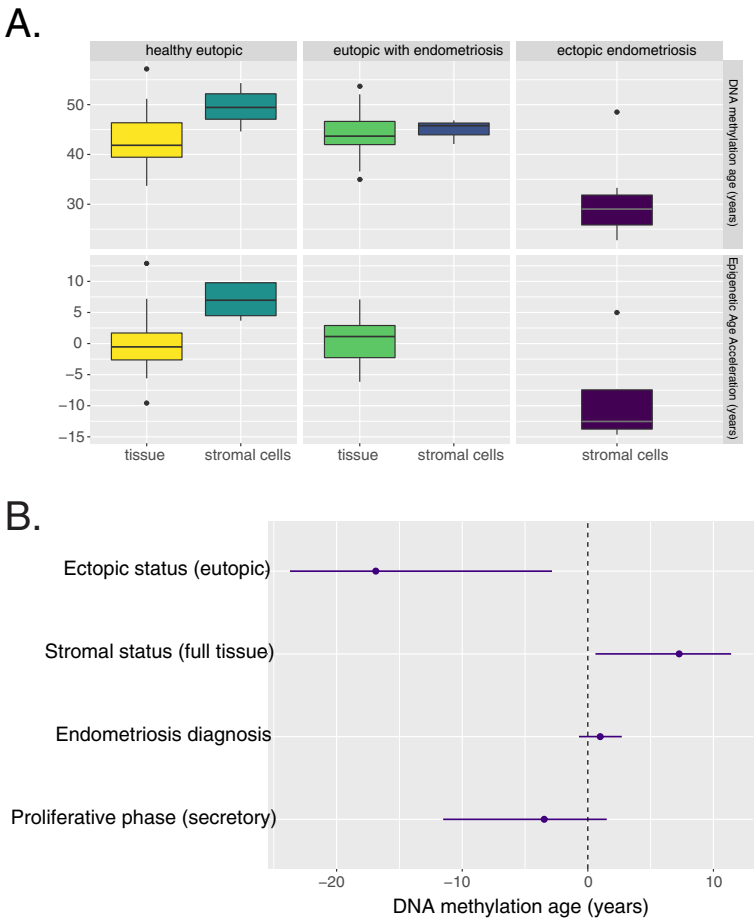

Figure 3. (**A**) DNA methylation age and Epigenetic Age Acceleration (EAA) of whole tissue as compared to isolated stromal cell populations. The age acceleration observed in stromal cells may be due to the effect of passaging. Healthy tissue: n = 34; Healthy stromal cells: n = 8; Eutopic tissue (endometriosis): n = 26; Eutopic stromal cells (endometriosis): n = 3; Ectopic stromal cells: n = 7. (**B**) Values of the coefficients estimated from the multivariate linear regression model; the point estimates are shown as points while the lines represent a bootstrapped 95% confidence interval. If the line crosses 0, the effect is not significant at 95% confidence. Age coefficient can be found in Fig. 1B.

be on average 6.57 years older than the whole tissue, with a 95% confidence interval estimate of 0.68–11.62, $p = 0.0138$. This age acceleration could be due to the effect of passaging, which has previously been shown to advance DNAm age[23]. Incorporating stromal status in the full model as a covariate did not change the effect size of ectopic status, indicating that while stromal samples may be older on average than whole tissue, this effect is independent of the age deceleration seen with ectopic status (Fig. 3B).

Cell migration does not affect DNA methylation age. To examine the effect of migration processes on DNAm age, we analyzed datasets of metastatic cancers as metastasis is a prototypical example of (malignant) tissue migration. Because it is known that cancer can dysregulate DNAm age, we confirmed the performance of Horvath's pan-tissue clock through the correlation between chronological age and DNAm age in the normal tissue samples: $r = 0.85$ ($p = 3 \times 10^{-10}$). As seen in Fig. 4A, cancer samples were more variable than normal tissues and exhibited age acceleration on average. However, the epigenetic age acceleration of metastatic tumor samples did not significantly differ from that of primary tumor samples on aggregate or in matched paired analyses, as can be seen in Fig. 4B by comparing the parameter estimates of primary tumors with metastases to metastatic tumors. This suggests that while the process by which a tissue becomes cancerous and invasive may accelerate its DNAm age, migration alone is not enough to affect DNAm age. The underlying mechanisms of cancer metastasis and endometriosis are not likely to be similar, so the deceleration in endometriosis further highlights the uniqueness of its disease process. These findings exclude the cell migration process or ectopic location as a contributor to DNAm age deceleration in ectopic endometriotic tissues.

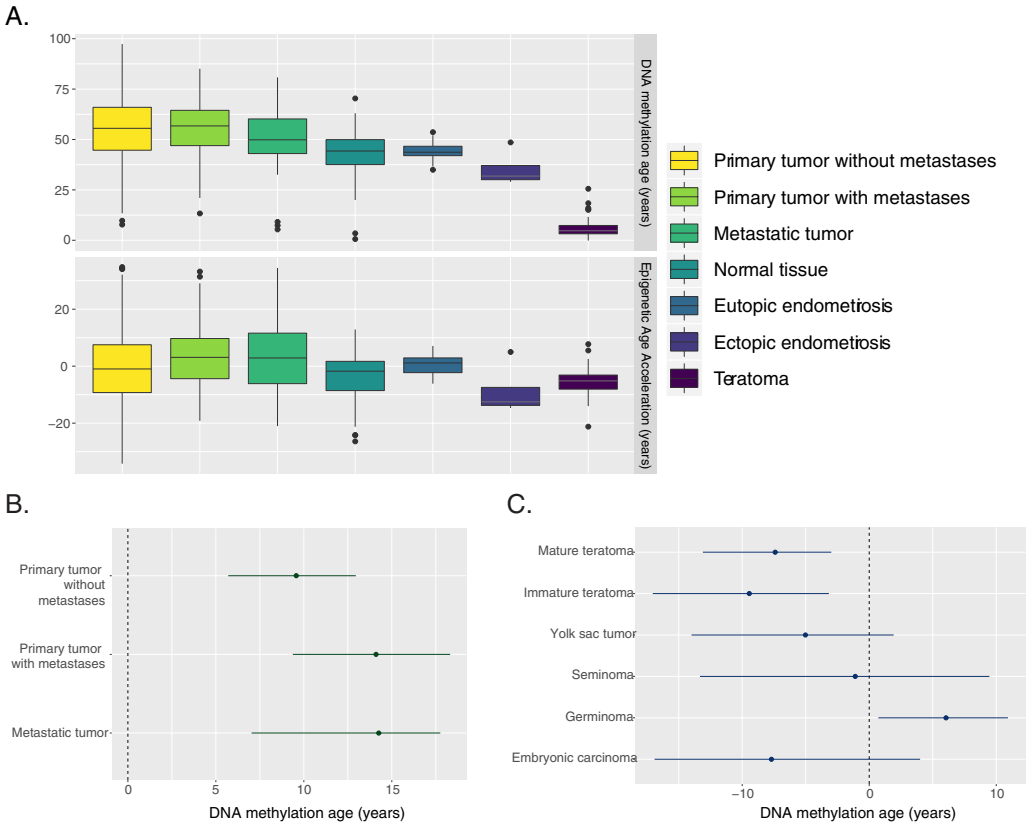

Figure 4. (**A**) Predicted DNA methylation age and epigenetic age acceleration by tissue type. Ectopic endometriotic tissue shows age deceleration comparable to that seen in teratomas. Primary tumor without metastases: n = 534; Primary tumor with metastases: n = 82; Metastatic tumor: n = 38; Normal: n = 81 (8); Eutopic endometriosis: n = 29 (3); Ectopic endometriosis: n = 7 (3); Teratoma: n = 25. Since EAA is dependent on knowledge of donor age, the samples for which the information about the age was absent were excluded from EAA analyses. These samples are given in parentheses. Limits of EAA plot exclude 23 outliers: 17 in primary tumors without metastases (3%), 5 in primary tumors with metastases (6%) and 1 in metastatic tumors (3%). (**B**, **C**) Values of the coefficients estimated from multivariate linear regression models; the point estimates are shown as points while the lines represent a bootstrapped 95% confidence interval. If the line crosses 0, the effect is not significant at 95% confidence. Age coefficients can be found in Fig. 1B.

Endometriotic stromal cells share a similar DNA methylation age pattern with multipotent tissues. Since cell migration, such as retrograde menstruation, does not explain the substantial DNAm age deceleration of ectopic tissue, we explored the possibility that ectopic tissue had been deposited during development and might share a similar DNAm age to tissues of developmental origin. Teratomas are benign growths of multipotent tissue that originate from developmental abnormalities, are always ectopically located, and are composed of undifferentiated cell types. We hypothesized that teratomas would exhibit DNAm age deceleration, but not as much as is observed in iPSCs or progenitor cells. The linear regression model we used to predict DNAm age of germ cell tumors given chronological age, tumor content, and indicator variables for tumor types was able to explain 75% of the variance in DNAm age. The presence of immature teratomas or mature teratomas decreased the predicted DNAm age by 9.44 and 7.40 years respectively ($p = 0.0016$ and $p = 0.0074$).

While the teratomas were predicted to have a very young DNAm age (Fig. 4A), this is explained by the fact that most of the samples came from young children (range 2 months–25 years, Fig. 1A). When adjusted for age with EAA, the difference is not as drastic (Fig. 4A) and the teratomas are more similar to ectopic endometriotic tissue. Interestingly, embryonic and placental germ cell tumors (embryonal carcinoma and yolk sac tumors) did not exhibit the age deceleration seen in mature and immature teratomas (Fig. 4C). Furthermore, none of the teratoma samples exhibited a DNAm of zero, as would be expected in fetal cells or iPSCs[5]. Taken together, this implies that tissue of a developmental origin exhibits epigenetic age deceleration that is not as extreme as is seen in fetal tissues or iPSCs, which is the pattern seen in ectopic tissue in endometriosis, and thus ectopic tissue in endometriosis may have a developmental origin.

Discussion

We show that epigenetic age estimators (e.g. the pan tissue epigenetic clock by Horvath) lend themselves to addressing vexing problems surrounding the etiology of endometriosis. Using human tissue samples, we demonstrate a) that DNAm age strongly correlates with chronological age in the endometrium, b) that ectopic tissue is substantially younger than eutopic tissue according to the epigenetic clock, c) that tissue migration through metastasis does not change DNAm age, and d) that ectopic endometriotic lesions and teratomas share age deceleration that is not as young as pluripotent embryonic or induced pluripotent cells according to the epigenetic clock.

Endometriosis is primarily a young woman's disease; symptoms often begin during puberty and the vast majority of cases resolve with menopause[24]. Senescence is a hallmark of aging, but it is also important for development and tissue homeostasis[25]. Endometriosis can be thought of as a problem of senescence because it is ultimately a problem of growth: endometriotic tissue should not grow and proliferate in ectopic sites, regardless of the exact process by which this growth occurs or is prevented in a non-diseased state. Therefore, it is possible that the natural process of aging causes senescence in ectopic tissues that had previously been resistant to apoptosis and necrosis. Using compounds to change DNAm age of ectopic lesions to one more consistent with a differentiated cell type may represent a therapeutic approach, akin to the success of differentiation therapy in leukemias[26].

Variability between lesions within person has been well-documented[16] and future research should investigate variability of DNAm age between lesions within an individual. While all of the lesions studied here were endometriomas, peritoneal disease presents with different colored lesions that are thought to represent a continuum from younger to older disease[27], a hypothesis that could be explored using DNAm age.

Limitations of this study include the use of datasets which only incorporated stromal cells from one type of ectopic endometriotic lesions across one stage of endometriosis (Stage III/IV), as well as the use of only one pathological process of abnormal cell migration (metastasis) to assess the unique DNAm age signature in endometriosis. As already highlighted, the ectopic samples were all periovarian, which may limit the generalizability of the findings, but ovarian endometriosis is the most common localization[28]. We also cannot rule out an effect of ovarian tissue DNA methylation age deceleration on ectopic endometriosis lesions due to non-cell autonomous effects. While we identified a DNA methylation age deceleration of 11 years in ovarian tissue when compared to endometrium (Supplementary Fig. S2), previous studies have shown that DNA methylation age is most likely a cell-autonomous process that is independent of the status of adjacent cells. For example, in the bone marrow, hematopoietic stem cell transplantation causes the DNA methylation age of recipient blood to reflect the donor's chronological age and not the recipient in age-discordant transplants[29]. In addition, while we did observe a large deceleration in age in endometriotic lesions in contrast to metastatic cancer lesions that retained the DNA methylation age of their associated primary tumors, we cannot rule out that there may be additional models of cell migration beyond cell migration associated with metastasis to be used to assess ectopic tissue localization. Finally, future availability of intact whole tissue ectopic lesion methylation datasets will provide an opportunity to reproduce the findings of this study.

Epigenetic clocks have potential utility as a prognostic indicator. Tissue of an embryonic origin may have a different clinical course than tissue resulting from retrograde menstruation and may necessitate different surgical or medical interventions. In order to validate this hypothesis, samples from women with known retrograde flow, such as that resulting from imperforate hymen, should be compared to those from affected men or women without a uterus. These disparate extremes may shed light into the etiologies of endometriosis and better methods of diagnostics, classification, and treatment.

Data availability

The datasets analyzed during the current study are publicly available from GEO and all accession numbers are listed in the supplemental information.

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Author contributions

K.L. designed the study, performed data analysis, and drafted the article. I.Y. contributed data. S.H. and J.A.M.A. provided support in interpreting the results. All authors made critical revisions to the manuscript and approved the final version.

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Competing interests

The authors declare no competing interests.

Additional information

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Chapter 2 : Decidualization-associated changes in epigenetic age are dysregulated in endometriosis and correlate with differences in DNA repair gene expression

Abstract

Background: Decidualization in humans is the process by which the uterus coordinates either embryo implantation or rejection. It involves a controlled sequence of gene expression and decidualization defects are associated with age-related reproductive decline. Aging affects gene regulation mechanisms, such as DNA methylation, in predictable ways. All cells in the body have a biological clock correlated with their chronological age, which we term epigenetic age, and is not variable in most tissues. Using a genetic approach, we explored the relationship between epigenetic age and decidualization with an existing dataset of hormone treated uterine cells from endometriosis patients resistant to decidualization and healthy controls.

Results: We found that given the same treatment, the decidualized cells had a decelerated epigenetic age while the non-decidualized cells did not. We found that expression of Fanconi anemia pathway genes *CORT*, *CENPX* and *FANCM* can be used to predict epigenetic age. Cortistatin is responsive to progesterone, the main driver of decidualization, and is co-expressed with *CENPS*. *CENPS* and *CENPX* together form a complex that binds to DNA and recruits *FANCM*, which initiates DNA repair. We further confirmed that *CORT*, *CENPX* and *FANCM* are differentially expressed by menstrual phase in controls, but the pattern is dysregulated in endometriosis. We also observed differences in gene expression of these genes by fertility status and in women with endometriosis.

Conclusions: Our findings demonstrate that there may be a role of the Fanconi anemia complex in fertility, in particular mediating the failure of decidualization in endometriosis.

Background

Decidualization is a key component of successful pregnancies. This process by which endometrial stromal cells terminally differentiate into decidual stromal cells regulates endometrial signaling to coordinate implantation of the blastocyst and initiate menstruation if the signal from the blastocyst is lost[33]. Defects of decidualization are common in both age-related reproductive decline[34] and endometriosis[35]. DNA methylation is one mechanism of regulating gene expression, with higher methylation resulting in reduced expression. DNA methylation also changes predictably with age and may be a mechanism of aging[36]. Biomarkers of this relationship between DNA methylation and age are called epigenetic clocks and deviations from expected epigenetic age have been correlated variously with disease and health outcomes as well as pluripotency[37].

Endometriosis is a common reproductive disorder that causes sub-fertility and pain. Endometrial stromal cells can be induced to decidualize in vitro, but endometrial stromal cells from persons with endometriosis typically fail to decidualize[35]. Lack of decidualization dysregulates the endometrium because secretions generated from the decidual cells are required to coordinate endometrial function[38]. The decidualization process consists of an acute pro-inflammatory phase (endometrial receptivity) followed by an anti-inflammatory phase (embryo development)[33]. If there is an absence of implantation, the subsequent decrease in progesterone triggers a second pro-inflammatory phase. Decidualized stromal cells within the endometrium secrete factors that initiate menstruation and are shed with the functionalis layer of the endometrium. If implantation is successful, a subset of stromal cells, the decidual stromal cells, migrate to surround the blastocyst and these migrated cells exhibit a greater potential for differentiation into decidualized cells[39]. Sub-fertility in endometriosis could be mediated by these

failures in decidualization[35], whether through the failure of both endometrial and decidual stromal cells to decidualize or through reduced number or capacity of decidualized decidual stromal cells. Our study demonstrates that endometrial stromal cells that decidualize in culture exhibit decelerated DNA methylation age while endometrial stromal cells from women with endometriosis that did not decidualize in culture do not. DNA methylation age is not correlated with markers of decidualization, but rather it correlates with expression of genes involved in DNA damage repair.

Methods

Data used were sourced from NCBI Gene Expression Omnibus (GEO) and each dataset is outlined below.

UCSF steroid hormone treated stromal cell lines (UCSF HT-EnSC)

This dataset comprises 12 endometrial stromal cell lines from 4 individuals with no uterine pathology, 4 with endometriosis stage I, and 4 with endometriosis stage IV[40]. Cell lines were selected for inclusion in the study based on their ability to decidualize, as confirmed by ELISA for IGFBP1, with the no uterine pathology samples confirmed to have decidualized in the E2+P4 condition and both the endometriosis stage I and stage IV samples having confirmed non-decidualization. Each cell line was treated with either estrogen (E2), progesterone (P4), both estrogen and progesterone (E2+P4), or vehicle, for a total of 48 samples. From these samples, DNA methylation was analyzed with the Illumina Infinium Methylation BeadChip array 450 (GSE130028) and gene expression was analyzed using the Affymetrix Human Gene 1.0 ST array (GSE145701).

UTU EndometDb

This gene expression dataset comprises 408 samples of whole tissue from healthy endometrium or peritoneum (53 controls) and endometriosis patient endometrium, peritoneum, and endometriosis lesions (115 patients)[41]. Gene expression was assessed using either the Illumina HumanH1-12 V4.0 expression beadchip or the Illumina HumanWG-6 v2.0 expression beadchip (GSE141549). For the purposes of this study, we used only the endometrium samples with known disease and menstrual phase status. The characteristics of this subset of the dataset can be found in Table 1.

Characteristic	Control, N = 33[1]	Stage I/II, N = 26[1]	Stage III/IV, N = 66[1]
age	38 (36, 42)	31 (27, 36)	31 (27, 38)
phase			
proliferative	7 (21%)	2 (7.7%)	15 (23%)
secretory	16 (48%)	9 (35%)	18 (27%)
menstruation	0 (0%)	3 (12%)	3 (4.5%)
medication	10 (30%)	12 (46%)	30 (45%)

Table 1 [1] Median (IQR); n (%)

AIIMS fertile and infertile endometriosis

This dataset comprises 36 samples of endometrial tissue from women without endometriosis (control) or with stage IV endometriosis[42]. Both control and endometriosis groups were further subdivided into either fertile or infertile groups, with 9 women in each of the four groups. Fertile controls were identified due to voluntary sterilization or hysterectomy for non-uterine disease reasons. Infertile controls were identified through the infertility clinic. Further information on the cohorts has been published. Data from GEO (GSE120103) had been log2 transformed and normalized to the 75th percentile, making them non-comparable to the other datasets used. We downloaded the raw expression data and used the normalizeBetweenArrays function from limma to log2 transform the data.

ACU recurrent pregnancy losses (ACU RPL)

This dataset comprises 72 samples of endometrial tissue from 24 patients with recurrent pregnancy loss (RPL), 24 patients with unexplained infertility (UI), and 24 controls. All participants were under the age of 35 and regularly cycling. Fertile controls had no relevant gynecologic or medical comorbidities and at least one live birth. Recurrent pregnancy loss was

defined as no successful pregnancies and two consecutive unexplained pregnancy losses of 20 weeks or less. Unexplained infertility cases were required to have at least 18 months of infertility duration. Further details of the inclusion criteria are available on GEO (GSE165004). Gene expression was assessed using the Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray.

ACU recurrent implantation failure (ACU RIF)

This dataset comprises 48 samples of endometrial tissue from 24 patients with recurrent implantation failure (RIF) and 24 fertile controls[43]. RIF was defined as no successful pregnancies after 3 or consecutive IVF cycles with at least 1 good quality embryo transfer in each cycle. More information on inclusion criteria can be found on GEO (GSE111974). Gene expression was assessed using the Agilent-039494 SurePrint G3 Human GE v2 8x60K Microarray.

Jackson Lab single cell

This single cell gene expression dataset comprises samples of endometrial biopsies that were dissociated into single cells from 9 endometriosis patients and 3 controls without endometriosis. The dataset that is available on GEO (GSE179640) also includes bulk RNA samples, samples from organoids, and ectopic samples, but these were not included in our analyses. Detailed information on the inclusion criteria, cohort characteristics, and sequencing methods has been published[44]. Of particular interest to our study is the fact that the majority of participants within this dataset were taking hormonal contraception and were not naturally cycling. We used the pre-processed h5ad file available from https://singlecell.jax.org/datasets/endometriosis-2022 for our downstream analyses, retaining the previously defined cell type clusters.

Analysis

DNA methylation age was calculated using Horvath's pan-tissue clock because variation due to tissue type or cell type composition is not expected[37]. Technical error has been measured at 3 years, so differences greater than 3 years were considered significant. Correlation was calculated using Pearson's product moment correlation. Weighted gene co-expression network analysis (WGCNA) was used to identify networks of co-expressed genes, which were then correlated with DNA methylation age, hormone treatment, and disease status[45]. For each phenotypic trait, modules with the greatest correlation coefficient were selected for follow up using DAVID[46,47]. Pathways identified by DAVID were selected based on both the corrected p-value and for having plausible connections to DNA methylation. Gene ontology terms were used with the annotation packages for each gene expression platform to identify probes belonging to the pathway. The platform used for gene expression, Affymetrix Human Gene 1.0 ST, provides expression as a probeset with resolution at either the gene level or the exon level. We fit linear models predicting DNAmAge from expression of probes in the pathway by their gene level expression or their exon level expression separately. Both the LASSO[48] and stepAIC[49] were used for variable selection. Expression of genes identified by the model were confirmed using the Mammalian Reproductive Genetics Database V2 available from https://orit.research.bcm.edu/MRGDv2[50]. Expression of genes identified by the model were evaluated for cell type specificity within the single cell data. Data was analyzed using Seurat.

To control for cell type proportions in bulk RNA expression datasets, we used CIBERSORTx to create a signature matrix file and deconvolute each of the datasets used[51]. We used a subset of the cells to create the signature matrix, consisting of just the control eutopic endometrium. The job parameters included disabling quantile normalization, kappa: 999, q-

value: 0.01, No. barcode genes: 300 to 500, Min. Expression: 1, Replicates: 5, Sampling: 0.5, and no filtering of non-hematopoietic genes from signature matrix during construction. With the signature matrix, we imputed cell fractions for each of the expression datasets used in our analysis. The job parameters included batch correction with B-mode source GEP file used for batch correction (created from the single cell dataset), quantile normalization not disabled (all datasets were microarray and this is recommended for RNA-seq data), absolute run mode, and 1 permutation. We used the online version of CIBERSORTx available at https://cibersortx.stanford.edu/.

We then added the sample level cell fractions as covariates to our linear regression models. The cell types imputed were myeloid, stromal, epithelial, lymphocytes, and endothelial. We dropped the smallest fraction, endothelial, to prevent overfitting.

Results

Decidualization is associated with decelerated epigenetic age

Aging is associated with changes in decidualization capacity and epigenetic age is an effective marker for establishing biological age. Previous studies have shown that epigenetic age is stable across tissues and mechanisms for modifying epigenetic age have not been established apart from those used for induced pluripotent stem cell reprogramming. We found that steroid hormone treatment of stromal cells in culture caused drastic changes in DNAmAge (Figure 2-1A) and that stromal cells that had decidualized in culture exhibited a decelerated DNAmAge, while the same cells in the vehicle, E2, or P4 conditions did not exhibit a significant change across persons (Figure 2-1B). These findings demonstrate that at least in this cell type, epigenetic age can be modified in response to hormonal treatment.

Markers of decidualization do not correlate with DNAmAge

We next wanted to explore if the process of decidualization itself was responsible for the observed epigenetic age deceleration. All of the samples that were confirmed to have decidualized by morphology and ELISA for IGFBP1 expressed transcripts for the decidualization markers *IGFBP1* and prolactin (*PRL*). Some of the samples that did not decidualize expressed intermediate levels of prolactin, so we tested the correlation between prolactin expression and DNAmAge to determine whether a partial decidualization phenotype might be associated with a partial decrease in DNAmAge. Expression of *PRL* and *IGFBP1* were highly correlated ($\rho = 0.88$, $p < 2 \times 10^{-16}$), but there was no significant correlation between expression of either *PRL* or *IGFBP1* and DNAmAge or the Age Acceleration Residual ($\rho = -0.25$, $p = 0.08$ for DNAmAge,and $\rho = -0.26$, $p = 0.12$ for Age Acceleration Residual). These

results suggest that despite the correlation of epigenetic age deceleration with decidualized cells, the process of decidualization is not causing this deceleration.

Expression of Fanconi anemia pathway genes can predict DNAmAge

As a correlate for changes in DNA methylation age with hormonal treatment, we explored expression signatures that could predict DNAmAge. Using modules of co-expressed genes, we found that the Fanconi anemia pathway is overrepresented among genes that correlated with estrogen treatment (correlation with estrogen: $\rho = -0.52$, $p = 0.0002$; overrepresentation of Fanconi anemia pathway $p = 2.1 \times 10^{-7}$). We then looked at all Fanconi anemia pathway genes identified by Gene Ontology term GO:0043240 that were expressed in the dataset to determine whether expression of these genes was predictive for DNAmAge. We first used probesets that mapped to genes, and then repeated the model fitting with probes that mapped to exons. Using both the LASSO and the stepAIC algorithm independently, we identified a subset of the pathway genes including *CORT*, *FANCM*, and *CENPX* as predictive of DNAmAge. Steroid hormone treatment and endometriosis stage were not selected by the LASSO or stepAIC for inclusion in the final model. The final linear model had DNAmAge as the dependent variable and as independent variables: log2 expression of *CORT*, *FANCM*, and *CENPX*, endometriosis status as a binary indicator, and age in years (Table 2). From these findings, we can conclude that expression of Fanconi anemia genes and disease status both correlate with epigenetic age.

Table 2

Independent Variables	Coefficient	Significance
CORT (log2 exp)	- 34.1	***
FANCM (log2 exp)	12.6	**
CENPX (log2 exp)	23.8	**

Endometriosis (yes/no)	4.6	*
Age (years)	1.7	***
R^2 = 0.68 Significance (p-value): *** 0-0.0009; ** 0.001-0.009; * 0.01-0.049		

Because the mapping for *CORT* did not distinguish between *CORT* and *CENPS*, a neighboring gene, we repeated the analysis with exon level mapping to determine which gene was driving the association. As shown in Table 3, *FANCM* and *CENPX* remained significantly associated and *CENPS* was the gene driving the association seen in the gene level model. Surprisingly, three exons in *FANCA* are highlighted here, even though *FANCA* was not chosen in the gene level model. *CENPX* changes its direction of association and endometriosis is no longer a significant covariate.

Table 3

Independent Variables	Coefficient	Significance
CENPS 7897637 (log2 exp)	- 6.3	**
FANCM 7974170 (log2 exp)	3.1	***
FANCA 8003515 (log2 exp)	3.2	*
FANCA 8003532 (log2 exp)	2.4	*
FANCA 8003541 (log2 exp)	1.4	*
CENPX 8019351 (log2 exp)	-9.8	**
Age (years)	0.8	***
R^2 = 0.81 Significance (p-value): *** 0-0.0009; ** 0.001-0.009; * 0.01-0.049		

Expression of Fanconi anemia pathway genes in female reproductive tissues

Having demonstrated a correlation between Fanconi anemia expression and DNAmAge in stromal cells, we next wanted to explore the expression pattern of these genes in endometrial and

other reproductive tissues. Using the Mammalian Reproductive Genetics Database V2 RNA-seq data of various female reproductive tract tissues, we demonstrate that the genes identified by our model are expressed in vivo and not just in the context of cultured cells (Figure 2-2). The dynamic pattern of expression across cell types and menstrual phases suggests a role for this pathway in the hormone-dependent regulation of endometrial function.

Single cell RNA-seq data

We next wanted to explore whether these genes were expressed in specific cell types within eutopic endometrium and if this cell type specificity differed in the context of endometriosis. The Jackson Lab dataset defined five cell types: endothelial, epithelial, lymphocytes, myeloid, and stromal (Figure 2-3A). Using a chi-square goodness-of-fit test, we tested whether cells with any expression of the linear model genes were equally likely to come from the five cell types. We found that expression of these genes is cell-type specific because cells positive for expression of these genes were not evenly distributed across the five cell types as would be expected if expression did not vary by cell type (Table 4). *FANCM* expression was higher in the eutopic endometrium from those with endometriosis than in control endometrium (Figure 2-3B), and both showed cell-type specific expression. *FANCA* expression was similarly higher in eutopic endometriosis endometrium than control with a disease associated enrichment in endothelial, myeloid, and stromal cells (Figure 2-3C), with cell-type specific expression seen in both control and endometriosis. *CENPX* expression was seen in more cells than *FANCM* or *FANCA*, with a similar pattern between control and endometriosis (Figure 2-3D), but a cell-type specific expression distribution was still observed. *CENPS* expression had similar levels overall in endometriosis and control but was more highly expressed in epithelial and stromal cells, with a noticeable enrichment in eutopic endometriosis endometrial stromal cells (Figure 2-3E). *CENPS*

expression is cell type specific in both control and endometriosis endometrium. Both *CORT* and *CENPS-CORT* expression is uncommon in cells from control and endometriosis endometrium (Figure 2-3F and 2-3G). While *CORT* expression seems to be cell-type specific in just control endometrium, but not cell-type specific in endometriosis endometrium, these differences could be due to low overall expression. The opposite pattern is seen with *CENPS-CORT* expression but may also be influenced by low overall expression.

Table 4

Gene	p-value in control endometrium	p-value in endometriosis endometrium
FANCM	1.051984e-16	1.224240e-46
FANCA	2.904296e-72	3.080426e-115
CENPX	7.865355e-91	9.500745e-180
CENPS	2.433222e-04	2.689909e-04
CORT	0.000000e+00	5.033871e-01
CENPS-CORT	1.486168e-01	9.720982e-03

CORT expression is downregulated in endometriosis and fertility, but upregulated in endometriosis-associated infertility

As part of our next analysis, we explored the pattern of expression of each of the identified genes to independently validate their association with endometriosis or infertility. We first examined the expression unadjusted through box plots, and then we performed a linear regression controlling for cell type proportions deconvoluted from single cell data. In the UCSF HT-EnSC dataset, the probe for *CORT* shares a mapping with the probe for *CENPS*, both located on chromosome 1. When we refit the model on the exon level, we found that *CENPS* is associated with DNAmAge, but we will explore expression of both *CORT* and *CENPS*. Additionally, *CENPS* and *CORT* form a fusion transcript, *CENPS-CORT*, and a fusion protein, *APITD1-CORT*, that has four isoforms. Across our validation datasets, some distinguish between

CENPS, *CORT*, and *CENPS-CORT* and some do not. We have designated the transcripts according to the location of the identifying probes.

In the UCSF HT-EnSC dataset, *CENPS* or *CORT* expression is decreased in the P4 condition as compared to the E2+P4 condition just in Stage I endometriosis, while no such changes are seen in the controls or Stage IV endometriosis (Figure 2-4).

In the UTC EndometDb dataset of whole endometrial tissue, *CORT* expression varies by menstrual phase in controls, with decreased expression during the secretory phase (Figure 2-5A). In contrast, endometrial tissue samples from women with endometriosis express consistent levels of *CORT* regardless of menstrual phase. *CENPS* shows no difference either by menstrual phase or endometriosis disease status and *CENPS-CORT* shows increased expression in the proliferative phase in endometriosis samples only (Figure 2-5B). When we control for cell fractions, there are no significant differences in *CORT*, but *CENPS* is decreased in Stage I/II endometriosis as compared to controls ($p = 0.007$).

In the AIIMS dataset, *CENPS* is significantly less expressed in infertile endometriosis as compared to infertile controls (Figure 2-6A). A probe in the *CORT* sequence that is shared with *CORT* and *CENPS-CORT* and annotated as a polyA site shows decreased expression in infertile controls as compared to fertile controls, but shows no difference in endometriosis (Figure 2-6B). Another probe in the shared *CORT*/*CENPS-CORT* sequence annotated as a preproprotein is significantly downregulated in fertile endometriosis as compared to fertile controls, with no differences between fertile controls, infertile controls, or infertile endometriosis (Figure 2-6C). After controlling for cell fractions, infertile controls had significantly decreased expression compared to fertile controls ($p =0.03$), fertile endometriosis patients had significantly decreased *CORT* expression as compared to fertile controls ($p = 0.03$), but infertile endometriosis patients

had significantly increased expression of *CORT* as compared to fertile controls ($p = 0.0002$). Infertile endometriosis patients also had significantly decreased expression of *CENPS* ($p = 0.02$).

In the ACU datasets, *CENPS* shows no difference between controls and recurrent implantation failure, recurrent pregnancy loss, or unexplained infertility (Figure 2-7). *CENPS-CORT* is downregulated in unexplained infertility as compared to controls or recurrent pregnancy loss (Figure 2-7). After controlling for cell fractions, unexplained fertility cases had significantly decreased expression of *CORT* ($p = 0.000003$).

Our findings suggest that *CORT* is expressed differently in the endometrium of individuals with endometriosis in response to hormones, that it has a different expression pattern across the menstrual cycle in controls as compared to individuals with endometriosis, that this differential expression is correlated with infertility, and that this correlation with infertility is unique to endometriosis and not observed with other forms of infertility. *CORT* expression is likely downregulated in infertility and endometriosis, but endometriosis-associated infertility is associated with an upregulation in *CORT*. *CENPS*, on the other hand, is downregulated in both endometriosis and endometriosis-associated infertility.

CENPX expression is dysregulated differently by endometriosis subtype

In the UCSF HT-EnSC dataset, *CENPX* is highly expressed in P4 treated control cells and downregulated in the E2+P4 treated Stage IV endometriosis cells, while Stage I endometriosis shows variable expression across the treatments (Figure 2-8A). In the UTU EndometDb dataset, *CENPX* is expressed more during the proliferative phase of controls than if the endometrium is not cycling due to use of hormonal contraceptives, while secretory expression is variable. Within endometriosis stage I/II, *CENPX* expression in the non-cycling endometrium is as variable as the secretory phase and is not less than the proliferative phase

expression. Within endometriosis stage III/IV, *CENPX* expression is at its highest during the proliferative phase with no differences between the secretory phase and the non-cycling endometrium (Figure 2-8B). In the AIIMS dataset, infertile controls express more *CENPX* than fertile controls, while infertile endometriosis express less *CENPX* than both fertile endometriosis and infertile controls (Figure 2-8C). After controlling for cell fractions, the fertile endometriosis patients had decreased CENPX expression as compared to controls ($p = 0.005$). In the ATU datasets, *CENPX* does not differ in expression between controls, recurrent implantation failure, recurrent pregnancy loss, or unexplained infertility. After controlling for cell fractions, the patients with recurrent pregnancy losses had increased expression of *CENPX* as compared to controls ($p = 0.0003$).

We conclude that *CENPX* shows differential expression patterns in individuals with endometriosis in response to hormone treatment and across the menstrual cycle, that it shows differential expression between fertile and infertile individuals with a different pattern in those with endometriosis, and that this differential expression is not seen in recurrent pregnancy loss or recurrent implantation failure.

FANCM expression is dysregulated in Stage I/II endometriosis

In the UCSF HT-EnSC dataset, *FANCM* expression increases with P4 treatment in the Stage I cells, with consistent expression in the control cells and variable expression in the Stage IV cells (Figure 2-9A). In the UTU EndometDb dataset, *FANCM* expression did not vary by menstrual phase in samples of control endometrium or samples from patients with stage III/IV endometriosis. Among persons with stage I/II endometriosis, *FANCM* was expressed significantly higher during the proliferative phase as compared to other phases (Figure 2-9B). In the AIIMS dataset, there were two probes for *FANCM*. Both showed increased expression in

infertile controls as compared to fertile controls and increased expression in fertile endometriosis as compared to fertile controls (Figure 2-9C). After controlling for cell fractions, the infertile controls expressed more *FANCM* than fertile controls ($p = 0.00003$), the fertile endometriosis patients expressed more *FANCM* than fertile controls ($p = 0.009$, while the infertile endometriosis patients expressed less *FANCM* than fertile controls ($p = 0.0004$). There was no significant difference in *FANCM* expression in the ATU datasets between controls, recurrent implantation failure, recurrent pregnancy loss, or unexplained fertility (Figure 2-9D).

Our findings show that *FANCM* shows differential expression in endometriosis, particularly in Stage I/II endometriosis, in response to hormone treatments and across the menstrual cycle. *FANCM* expression is differential by fertility status in endometriosis, but these differences are not seen in recurrent pregnancy loss or recurrent implantation failure.

Discussion

We have identified that DNA methylation age is modifiable given hormone treatment in endometrial cells and that decidualization decelerates DNA methylation age significantly. This deceleration correlates with expression of Fanconi anemia pathway genes. Both the DNA methylation age and expression of the identified genes are dysregulated in endometriosis. We have independently validated differential expression of the Fanconi pathway genes *CORT*, *CENPS*, *CENPX*, and *FANCM* in four additional datasets of gene expression data from endometrial tissue from individuals with endometriosis or infertility. We found that in addition to the differential expression by endometriosis status in response to hormone treatment originally identified, expression of these genes differed in their pattern across menstrual phases between those with and without endometriosis. Furthermore, differences were seen by fertility status in endometriosis that were not present in recurrent pregnancy loss or recurrent implantation failure. CENPS, CENPX and FANCM, which were identified in our final linear model, form a complex that stabilizes DNA and recruits the rest of the Fanconi Anemia core complex. Expression of these DNA repair genes may be important for DNA methylation reprogramming, for the function of decidualization, or may be specifically dysregulated in endometriosis.

DNA methylation reprogramming canonically occurs during fertilization and development of primordial germ cells. It also occurs during the in vitro process of inducing pluripotency in somatic cells and as confirmed here, in response to steroid hormone treatment in endometrial cells. The differences seen in expression of Fanconi anemia pathway genes associated with DNA methylation age deceleration may indicate that DNA repair is important for maintenance or reprogramming of DNA methylation. DNA damage is more likely to occur during replicative stress caused by frequent proliferation, as in the case of rapidly dividing

cancer cells[52]. Uterine remodeling requires the entirety of the functionalis layer of the endometrium to regrow from the basalis layer with each menstrual cycle. Homologous recombination is one way in which DNA damage is repaired and often results in the loss of methylation markers[53]. Another possibility is that the Fanconi anemia complex is involved in DNA methylation programming beyond its involvement in DNA damage repair.

Decidualization is a necessary process for endometrial function, and it is possible that the genes identified in this study are involved in decidualization. CORT is a neuropeptide that was first identified in the cortex but is known to be expressed in many other tissue types[54]. It is a target of the progesterone receptor B in endometrial cell lines[55] and is required for prolactin release[56], which is a biomarker of decidualization. Its proximity to CENPS or the CENPS-CORT readthrough transcript could be important for upregulating the Fanconi anemia complex and preventing DNA damage during the endometrial remodeling of decidualization. Further research on different timepoints in the decidualization process could provide insight into the mechanisms of both the DNA methylation reprogramming and DNA damage repair or prevention involved.

Finally, the genes we have identified may be involved in the disease process of endometriosis. Throughout the validation datasets, we have seen different expression patterns of these genes in endometriosis as compared to both controls and other forms of infertility. It is possible that the disease process of endometriosis changes how the Fanconi anemia pathway affects infertility since the infertile controls and other non-endometriosis forms of infertility do not share the same expression patterns, with fertile endometriosis sharing expression patterns with infertile controls. We have validated differential expression of these genes across different endometriosis datasets, but the non-endometriosis infertility datasets did not validate differential expression, suggesting that dysregulation of these genes is specific to endometriosis.

Understanding the mechanisms of failed decidualization in endometriosis could provide targets for improved fertility, but improved understanding might also shed light on DNA methylation reprogramming beyond the context of development, on the general function of decidualization, and on the disease process of endometriosis.

Figure 2-1

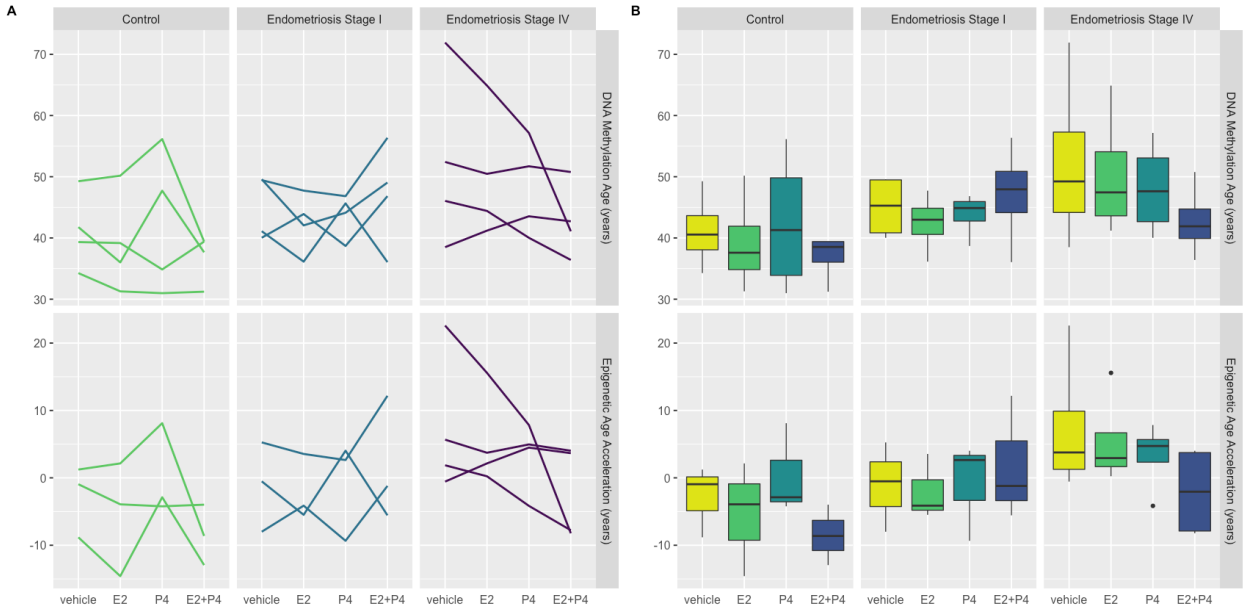

Figure 2-1: Treatment with steroid hormones changes DNA methylation age

A: Effect of steroid hormone treatment in cultured stromal cells on DNA methylation Age (DNAmAge) and Epigenetic Age Acceleration (EAA); two samples are missing from EAA because donor age is unknown. Hormone treatment was done concurrently, connected lines indicate the same donor and not a timecourse.

B: E2+P4 condition decreases EAA in control cells only; Stage IV endometriosis cells have variable EAA in the E2+P4 condition but not significantly different.

Figure 2-2

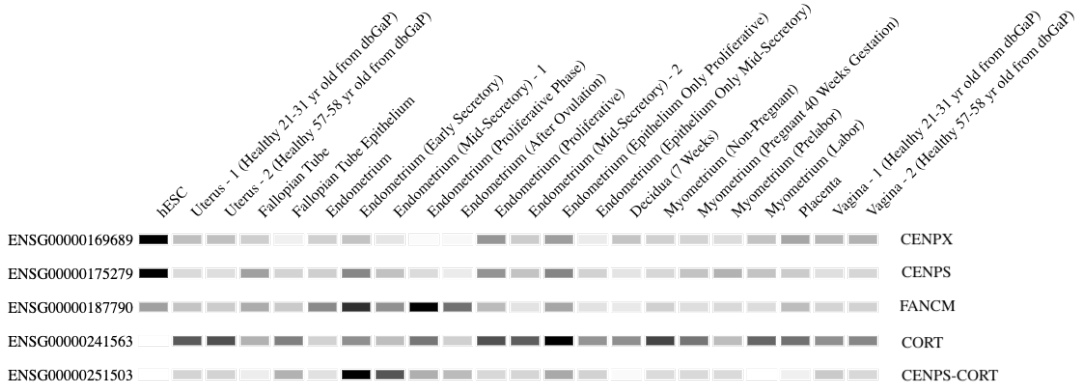

Figure 2-2: Fanconi anemia pathway genes are expressed in female reproductive tissues

RNA-seq data represented as digital PCR heatmap showing relative expression of the listed transcripts.

Figure 2-3

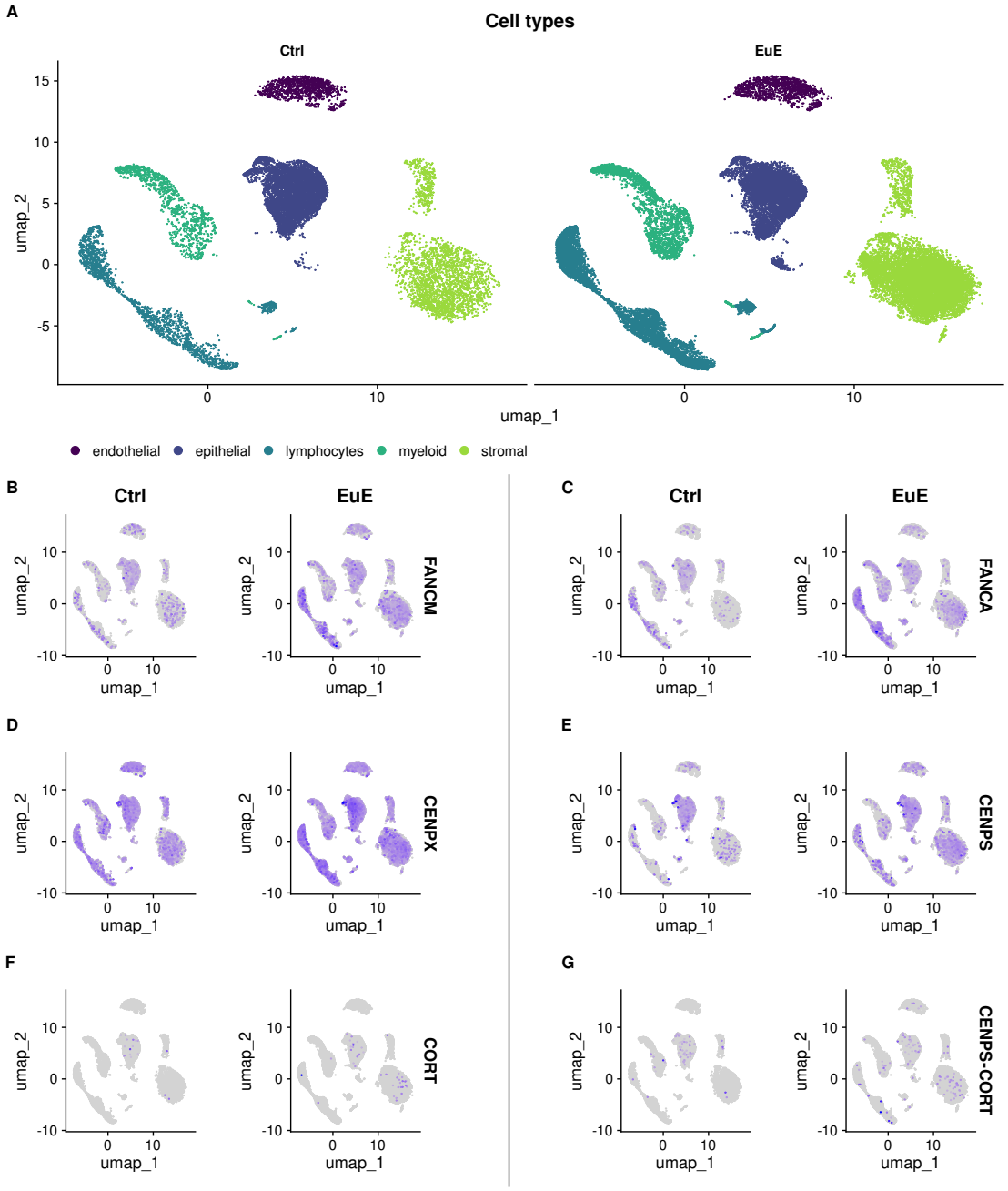

Figure 2-3: Fanconi anemia pathway genes are expressed differently by cell type in the endometrium

A: Uniform Manifold Approximation and Projection (UMAP) plots of the single cell dataset split by sample type and labeled by cell type showing distinct separation of cell types. Ctrl indicates endometrium sample from a person without endometriosis (3 donors). EuE indicates eutopic endometrium sample from a person with endometriosis (9 donors).

B-G: Expression overlaid on top of UMAP plots for each of the 6 genes of interest. Data was plotted with the most highly expressing cells on top to show location of gene expression due to the small number of cells expressing certain genes. Cell type specific expression is evident, but oversampling of endometriosis makes direct comparison more difficult.

Figure 2-4

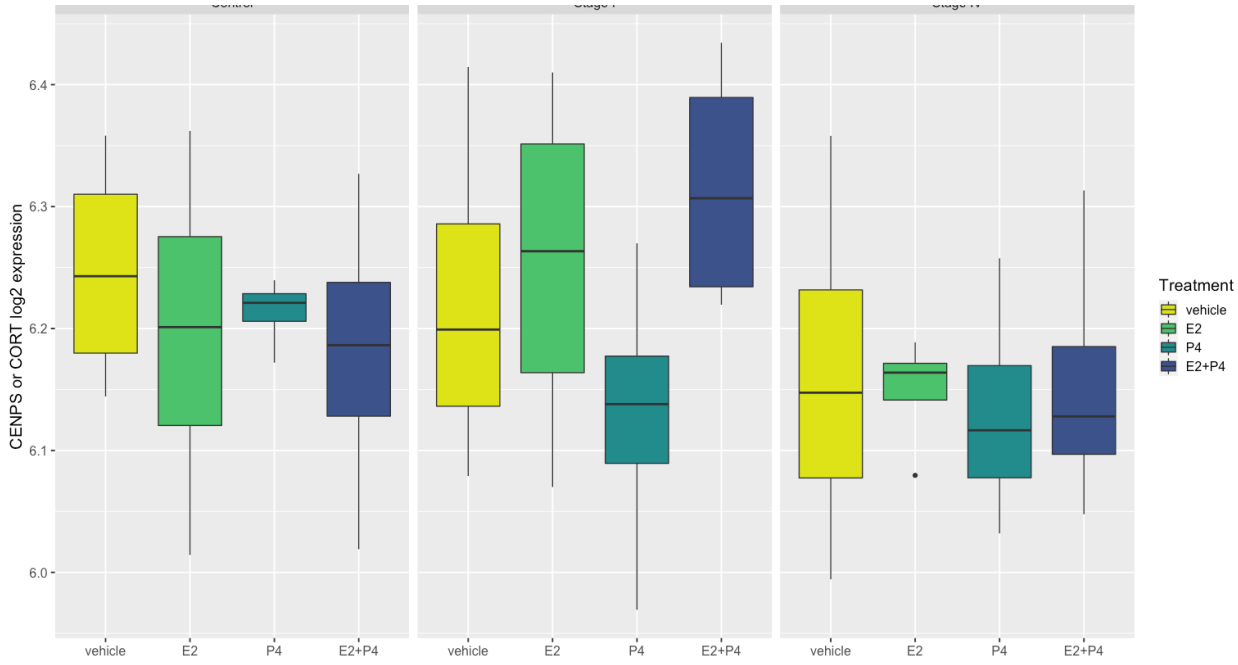

Figure 2-4: CENPS or CORT expression is differentially expressed in response to progesterone in endometriosis (UCSF HT-EnSC)

Stromal cells from the eutopic endometrium treated in culture with vehicle, estrogen (E2), progesterone (P4), or estrogen and progesterone (E2+P4). Array does not distinguish between CENPS or CORT; probe shows decreased expression with P4 in both Stage I and Stage IV endometriosis. Stage I endometriosis show increased expression with E2+P4

Figure 2-5

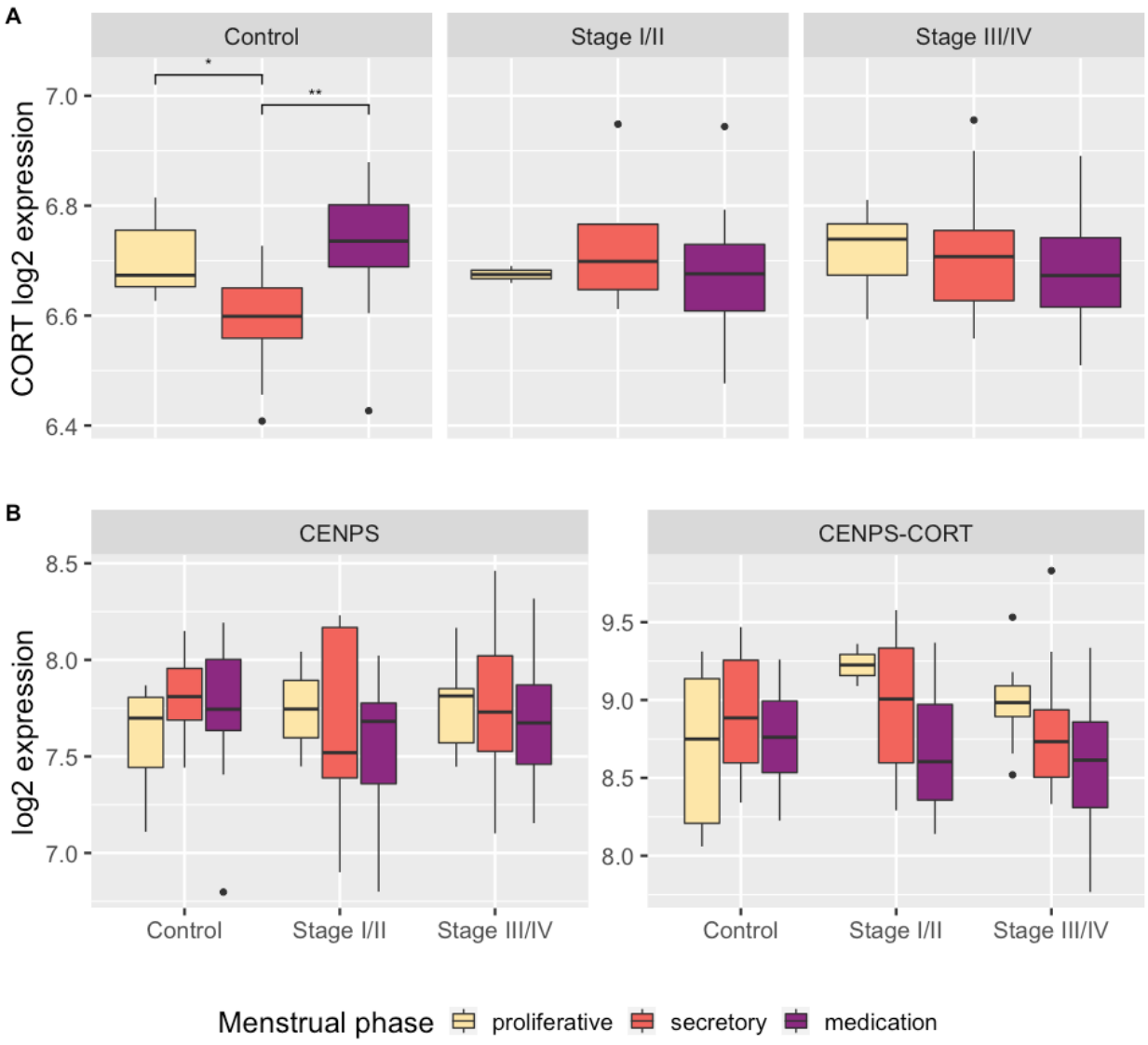

Figure 2-5: CORT expression is differentially expressed by menstrual phase in controls but not in endometriosis (UTU EndometDb)

A: Differential expression by menstrual phase is seen in CORT expression in controls (whole tissue endometrium), but the same pattern is not seen in endometriosis Stage I/II or Stage III/IV

B: CENPS shows no difference in expression either by menstrual phase or disease status. CENPS-CORT is upregulated in the proliferative phase as compared to medication in just endometriosis.

Figure 2-6

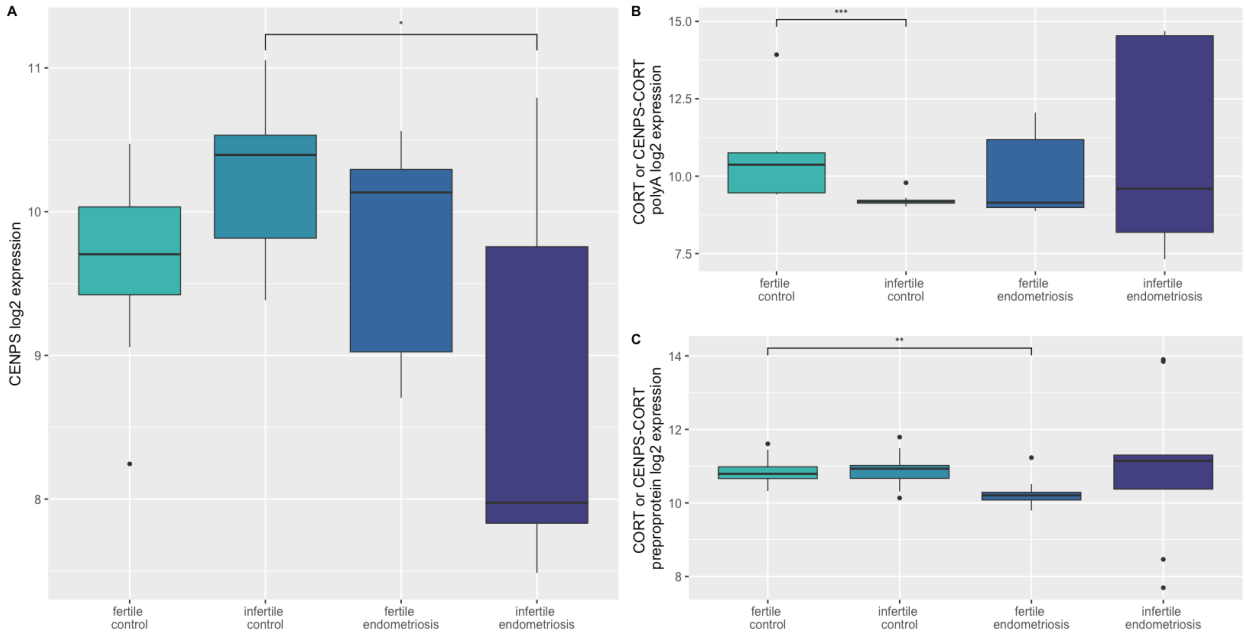

Figure 2-6: CENPS and CORT/CENPS-CORT are differentially expressed by fertility and endometriosis status

A: CENPS is downregulated in infertile endometriosis as compared to infertile controls (whole tissue endometrium). Endometriosis samples show more variable expression than controls.

B: A probe that maps to a polyA annotated site in both CORT and CENPS-CORT sequences is downregulated in infertile controls as compared to fertile controls, but is much more variably expressed in infertile endometriosis.

C: A probe that maps to a site annotated as preproprotein in both CORT and CENPS-CORT sequences is downregulated in fertile endometriosis as compared to fertile controls, but shows similar expression between fertile controls, infertile controls, and infertile endometriosis.

Figure 2-7

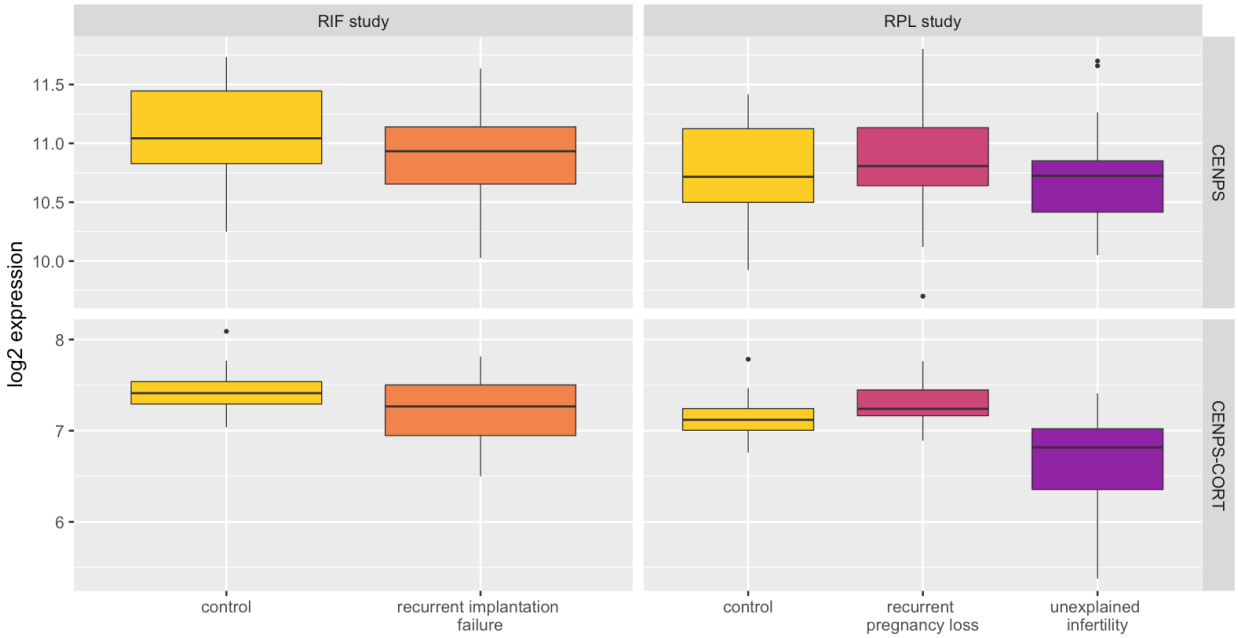

Figure 2-7: CENPS is not differentially expressed in RIF or RPL, but CENPS-CORT is downregulated in unexplained infertility

CENPS shows no differential expression in recurrent implantation failure (RIF), recurrent pregnancy loss (RPL), or unexplained infertility (UI). CENPS-CORT is downregulated in unexplained infertility as compared to controls or recurrent pregnancy loss.

Figure 2-8

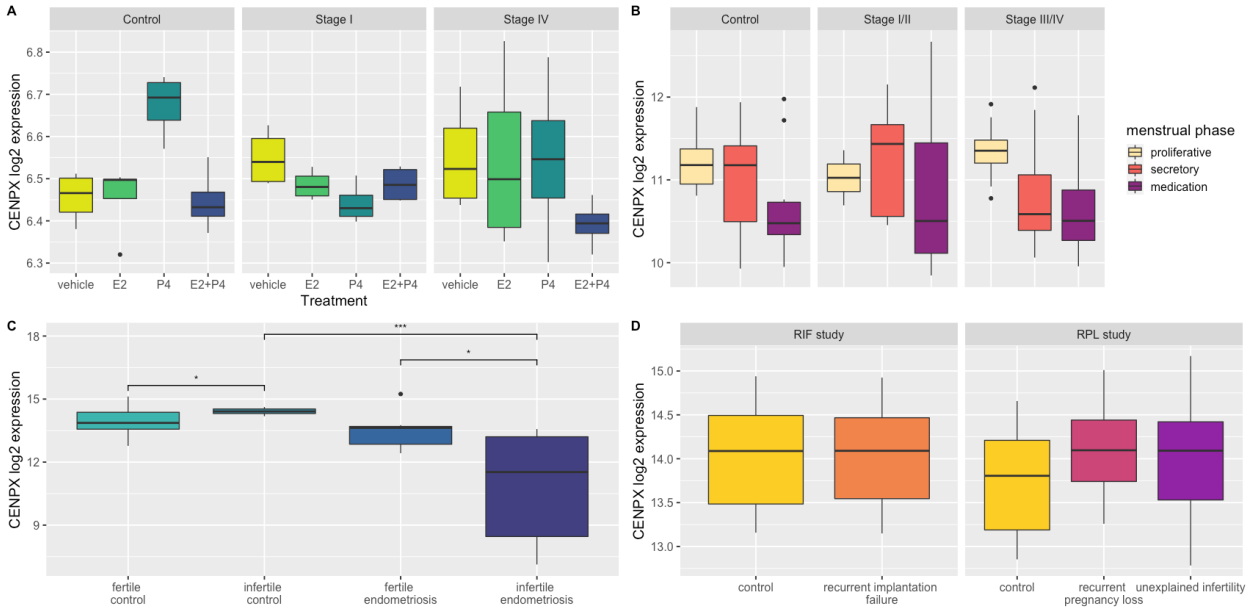

Figure 2-8: CENPX expression is dysregulated in endometriosis

A: Endometrial stromal cells from controls show upregulated CENPX expression when treated with P4, but this is not seen in endometriosis, with Stage I having greatest expression in vehicle and least in P4 and Stage IV having variable expression across samples and the least expression in E2+P4.

B: Whole endometrium tissue from controls has CENPX upregulated in the proliferative phase as compared to medication, with variable expression in the secretory phase. Stage I/II endometriosis has variable expression in both secretory and medication phases. Stage III/IV endometriosis has highest expression in the proliferative phase, with secretory and medication both having lower expression of CENPX.

C: CENPX expression is differential between fertile and infertile groups with opposite directions of effect by endometriosis status. Infertile controls have more CENPX expression as compared to fertile controls, but infertile endometriosis samples have decreased CENPX expression when compared to fertile endometriosis and infertile controls.

D: No differences in CENPX expression is seen in RIF, RPL, or UI.

Figure 2-9

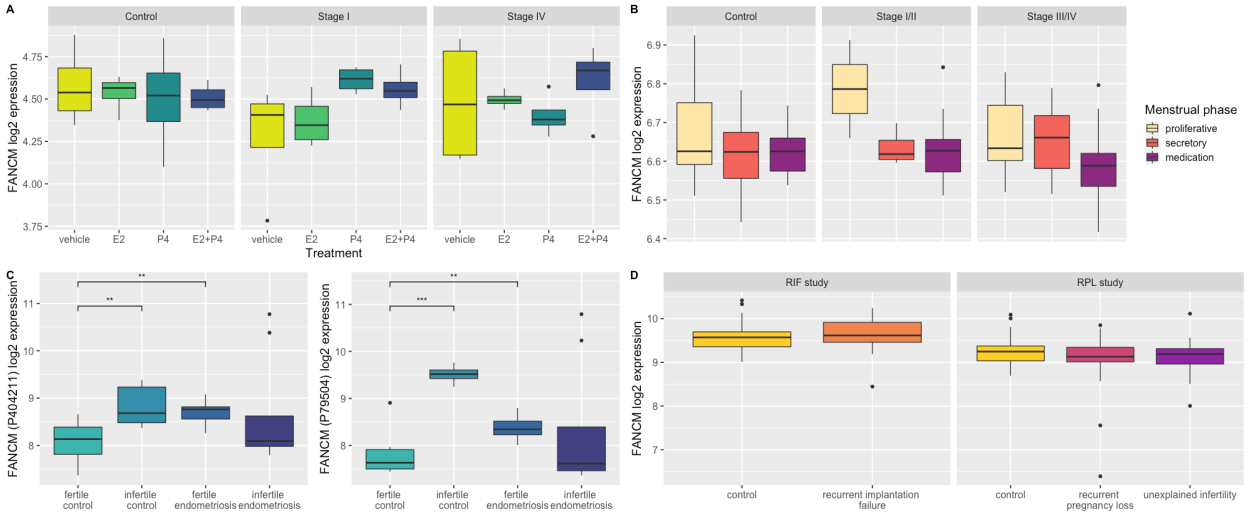

Figure 2-9: FANCM expression is affected by menstrual phase in endometriosis and is upregulated in fertile endometriosis

A: FANCM expression is unchanged by steroid hormones in control cell lines, but is upregulated with P4 or E2+P4 treatment in Stage I endometriosis and with E2+P4 treatment in Stage IV, as well as more variable expression with vehicle.

B: FANCM is upregulated in the proliferative phase in Stage I/II endometriosis, but is consistent by menstrual phase in controls and Stage III/IV endometriosis.

C: FANCM has two probes in the AIIMS dataset, but they show the same pattern of expression with differing magnitudes. FANCM is upregulated in infertile controls as compared to fertile controls, but it is also upregulated in fertile endometriosis as compared to fertile controls. Infertile endometriosis has the most variable FANCM expression.

Chapter 3 : Differences in gene expression and signaling in menses-derived stem cells (MenSCs) amongst individuals with NSAID-resistant or NSAID-responsive menstrual pain

Introduction

Endometriosis is a common reproductive disease that has a prevalence comparable to that of diabetes, around 10%, but lacks effective treatment for both management of symptoms and recurrence of disease[57,58]. Among those with symptomatic endometriosis, two thirds report their symptoms began in adolescence[59] and up to 70% of adolescents presenting with chronic pelvic pain resistant to treatment have stage I/II endometriosis[60]. However, while endometriosis is defined by the presence of uterine-like tissue outside of the uterus[57], there is limited evidence to suggest that this ectopic tissue causes the pain experienced by patients[61]. People with endometriosis-associated pain have a lower quality of life and are less likely to be employed than those with asymptomatic endometriosis or healthy controls[62,63].

Menstrual pain is experienced by a majority of people with symptomatic endometriosis[59,64] and it is thought to be initiated by prostaglandins that cause uterine contractions which in turn cause pain through mechanical and hypoxemic mechanisms[65,66]. Popular over-the-counter (OTC) painkillers work by inhibiting the enzyme that produces prostaglandins, cyclooxygenase (COX)[65]. Many people with endometriosis do not experience adequate pain relief from OTC painkillers for their pelvic pain, and pain remains one of the main challenges in managing and treating this condition[61].

One of the challenges in studying endometriosis is the dependence not only on surgically obtained tissue samples, which increases the risk and cost involved, but also on surgically diagnosed endometriosis, which introduces selection bias due to access to medical care and the average delay to diagnosis[67]. Menses can be collected noninvasively and contains stem cells that can be isolated and studied, termed MenSCs[68,69]. Cultured MenSCs from people with endometriosis are known to overexpress COX-2, the enzyme that produces prostaglandins and

the target of non-steroidal anti-inflammatory drugs (NSAIDs)[70]. NSAID-resistant dysmenorrhea is menstrual pain that is not improved after taking NSAIDs and it is found in 18% of people with dysmenorrhea[71], but in 90% of people with endometriosis-associated pain[64]. Severity of menstrual pain and its response to painkillers are clinical predictors of endometriosis that can be assessed in populations that have not been evaluated for endometriosis.

Previous research studies across diverse cohorts have highlighted an association between dysmenorrhea and clinical characteristics of menstrual flow, such as duration of bleeding and the presence of heavy bleeding or clots[15,72–76]. Quantitative research on menstrual flow has focused on blood loss specifically instead of the entirety of the heterogenous menstrual tissue that was shed[77]. Reports on menstrual bleeding consistently find large amounts of variation: 1) between individuals, 2) between different menstrual cycles from the same individual, and 3) between the perceived heaviness of a period and its measured heaviness[78]. However, 1) individuals can be categorized either as having light/normal periods or as susceptible to heavy periods and generally do not fluctuate between very light periods and very heavy periods[79], 2) most of the variation between periods is observed in people with very heavy periods[77], and 3) menstrual flow per period tends to fit a uniform distribution so it is unlikely that a single sample will misclassify a person as a light vs heavy bleeder[80]. We, therefore, would like to propose that the relationship between dysmenorrhea and heavy or prolonged menstrual bleeding is related to increased amounts of endometrial tissue, likely caused by activation of growth signaling pathways.

The PI3K-Akt-mTOR growth signaling pathway is responsible for communicating nutrient availability to cells and tissues and controlling cellular or tissue growth in response[81]. Endometriosis is a condition where cells grow in situations they should not grow and the PI3K-Akt-mTOR pathway has been implicated in endometriosis[82–84]. In addition to its role in nutrient

sensing, the mammalian target of rapamycin (mTOR) is a regulator of cell growth and proliferation[85] and is known to be upregulated in ectopic endometriotic tissue[83]. Inhibition of Akt, upstream of mTOR in the PI3K-Akt-mTOR pathway, has recently been shown to suppress the growth and survival of endometriotic cells, in conjunction with ERK 1/2[84]. Furthermore, the target of NSAIDs, COX-2, can be connected to the PI3K-Akt-mTOR pathway. Prostaglandins have long been implicated in dysmenorrhea and endometriosis[86,87] and are generated by COX-2[88], which is upregulated in ectopic and eutopic endometriotic tissue[89], as well as in MenSCs derived from individuals with endometriosis[70]. Evidence exists that PGF2a can stimulate PI3K/ERK/mTOR signaling[90] and that PI3K can regulate COX-2 expression[91]. We hypothesize that MenSCs from individuals with NSAID-resistant pain may demonstrate altered growth signaling and this might be mechanistically related to their pain.

In this study, we have collected menses from three groups of individuals: those with no menstrual or pelvic pain, those with menstrual pain that does not respond to NSAIDs, and those with menstrual pain that does respond to NSAIDs. We have isolated MenSCs from these participants and found differences by pain phenotypes in menstrual flow rate and abundance of MenSCs in the menstrual effluent. While we do not find expression of COX-2 to correlate with the presence or absence of menstrual pain or its responsiveness to NSAIDs, we observe a marked difference in the expression of stem cell marker NANOG by pain status. Finally, we demonstrate a differential response to starvation stress in MenSCs by pain status of individuals.

Materials and Methods

This study is a case-control study measured cross-sectionally with a primarily categorical exposure. We defined three groups characterized by their medical histories: one group has no history of dysmenorrhea or any other pain syndromes; another has moderate/severe NSAID-resistant dysmenorrhea; and the third has moderate/severe dysmenorrhea that responds well to NSAIDs.

Participants

Institutional review board approval was obtained and participants were recruited primarily through direct emails sent by the UCLA registrar's office to students identified by the school as female and between the ages of 18-26. All participants recruited were required to have a menstrual flow heavy enough to donate menses regardless of whether cycling was natural or hormone-induced. Moderate to severe pain was defined as moderate: daily activities affected, analgesics required for normal functioning; and severe: analgesics ineffective and activity inhibited. Healthy controls were screened for no history of menstrual pain and no history of pain with defecation, urination, or intercourse. Participants with NSAID-responsive pain were required to have at least moderate pain that resolved completely upon taking NSAIDs. Exclusion criteria for all three groups included current vaginal or urinary infection, including yeast infections or sexually transmitted infections, current use of an IUD, history of endometrial, cervical, or ovarian cancer. Participants were screened for exclusion criteria prior to scheduling a study visit and were fully consented.

Of 45 participants enrolled in the study, 25 donated menses samples and 11 donated a second sample for a total of 36 samples. Eight people with no pain donated 12 samples, 14 people with

NSAID-resistant pain donated 19 samples, and 3 people with NSAID-responsive pain donated 5 samples.

Questionnaire

The study questionnaire was self-administered through REDCap and is a modified version of the World Endometriosis Research Foundation Endometriosis Phenome and Biobanking Harmonisation Project (EPHect) clinical questionnaire[92] with added questions on gastrointestinal/bladder pain and function.

Study visits and sample collection

Visits were scheduled on the second day of the participant's menstrual cycle or breakthrough bleeding. After arriving to the study site, participants were provided a menstrual cup (Diva International) and instructed on how to self-insert the menstrual cup in a private, single-occupancy restroom. Depending on the participant's perceived maximal menstrual flow, collections were either a) overnight for 12 hours, b) during the day for 12 hours, or c) during the day until the cup reached capacity (30 mL). At the end of the collection, participants removed the cup wearing gloves and transferred the contents to a urine specimen cup. They were instructed to ensure no residual tissue remained in the cup and were provided a squeeze bottle containing isolation media (2.5 μg/mL amphotericin B, 1% PSN, 0.5 mM EDTA in PBS) to dislodge any remaining material in the cup. Samples were immediately weighed, photographed, and placed in the fridge prior to processing (maximum of 24 hours). After donating the sample, participants completed a short questionnaire on pain and NSAID use during the collection.

Participants were permitted to donate a second time and were instructed to insert the menstrual cup 12 hours prior to donating the sample. Some participants had very short menstrual cycles that did not yield much menstrual flow on the second day of their periods and thus began the

second collection shortly after their bleeding started, providing an earlier sample than their first. Participants with a heavy menstrual flow tended to give a shorter second collection once they were acquainted with the capacity of the cup.

MenSC isolation

Menses samples were processed using an adapted version of a published protocol (Tavakol et al 2018). Briefly, menses samples were diluted in isolation media prior to separation in Ficoll-Paque. Samples frequently contained large pieces of intact tissue where were processed separately with a collagenase treatment. The tissue fragments were dropped into a 50 mL Falcon tube and the remaining liquid suspension was overlaid over Ficoll-Paque and centrifuged for 20 minutes at 400 *x g*. The tissue fragments were diluted in HBSS with added calcium and magnesium and then put into a 6 well plate, either dropwise with a serological pipette or by transfer to separate wells with a P1000 micropipette. Tissue fragments were submerged in 2-5 mL HBSS with calcium and magnesium 100 U/mL collagenase

II with 1% PSN and 2.5 μg/mL amphotericin B and incubated at 37°C with 5% humidified CO_2 overnight (12-24 hours). After the collagenase treatment, the cell solution was treated identically to the turbid interphase of the Ficoll-Paque gradient.

The turbid interphase or collagenase treated cell suspension were pelleted by centrifugation (1000 rpm for 5 minutes) and washed with PBS until the cell pellet was compact. The cell pellet was resuspended in 1 mL cell pellet resuspension media (DMEM/F12 with no phenol red, 10% FBS, 0.1 mM non-essential amino acids and 2 mM L-glutamine). The cell suspension was transferred to a T25 vented flask with 2.5 mL pre-warmed plating media (DMEM/F12 with no phenol red, 15% FBS, 1% PSN, 1% L-glutamine, and 1 μg/mL amphotericin B). After 24 hours incubation at 37 °C in a humidified 5% CO2 incubator, 3 mL plating media was slowly added to

the flask. After 2 days, cell culture media was aspirated from the flask; 3 mL PBS was added to the flask and then aspirated; and then 5 mL growth media (DMEM/F12 with no phenol red, 10% FBS, 1% PSN, and 1% L-glutamine) was added to the flask.

Cell culturing

Cells were grown in culture with growth media and culture media was changed every 3-5 days. Cells were grown to 80-90% confluence and then passaged using a pre-wash with PBS and TrypLE treatment for 5 minutes. Cells were especially adherent and required the use of a cell scraper to passage. Cells were frozen at each passage in freezing media (10% DMSO in growth media) and frozen with a Corning CoolCell at -80 °C.

RT-PCR

RNA was extracted using Quick-DNA/RNA Microprep Plus Kits (Zymo D7005) according to manufacturer specifications. RNA was quantified using a NanoDrop spectrophotometer and 300 ng of RNA per sample was used to make cDNA using the SuperScript III First-Strand Synthesis SuperMix system (Invitrogen 18080-400, Thermo Fisher Scientific). DreamTaq Green PCR Master Mix (ThermoScientific K1082) was used to amplify sequences matching specific primers. Primer sequences are given in Table 5.

Table 5

Gene		Sequence
COX-2	Forward	AGAAAACTGCTCAACACCGGA
	Reverse	ACCGTAGATGCTCAGGGACT
NANOG	Forward	ACCAGTCCCAAAGGCAAACA
	Reverse	AACCTCGCTGATTAGGCTCC
SOX-2	Forward	AACCAGAAAAACAGCCCGGA
	Reverse	GTTCATGTGCGCGTAACTG
OCT-4	Forward	TCCCTTCGCAAGCCCTCATT
	Reverse	TCCCTTCGCAAGCCCTCATT

TBE gels with 1.25% agarose and SYBR Safe DNA gel stain (ThermoFisher Scientific) were cast and 15 uL of PCR products in master mix were run using the Mini-Sub Cell GT

Electrophoresis Cell system (Bio-Rad). Molecular weights were confirmed with 10 uL of TrackIt 1 Kb Plus DNA Ladder (Invitrogen 10488085, ThermoFisher Scientific).

Starvation experiments and immunocytochemistry

Frozen aliquots were thawed from passage 3 by placing the cryovial with 1 mL cell suspension in pre-warmed water at 37°C and then transferring to 4 mL pre-warmed growth media in an Eppendorf tube. Diluted cell suspensions were centrifuged at 1000 rpm for 5 minutes, supernatant was aspirated, and the cell pellet was resuspended in 1 mL of growth media before transferring to a T75 vented flask with 10 mL pre-warmed growth media. Media was changed every 3-4 days and once cells reached 70-80% confluence, they were passaged and split 1:4. At the next passage, cells were counted using a Countess II and seeded on glass coverslips in a 12 well plate at 70,000 cells per well. After two days, the media was exchanged with either growth media (DMEM/F-12, no phenol red, ThermoFisher 21041, supplemented with 10% FBS and 1% L-glutamine) or starvation media (DMEM, no glucose, no phenol red, no glutamine, ThermoFisher A14430, without supplements). Full details of the media formulations are available from the manufacturer, but notably the starvation media lacks glucose and key amino acids, including glutamine, alanine, asparagine, aspartic acid, cysteine, glutamic acid, and proline. After a 24 hour starvation period, coverslips were fixed in 3.7% formaldehyde in PBS for 20 minutes, then blocked in 10% normal goat serum in PBST (0.4% Triton-X in PBS) and stored in 4°C. Coverslips were washed by gentle submersion in PBST 3 times and dried by a quick touch to a kimwipe, then washed 3 times in ddH_2O and dried the same. Staining was done by placing the coverslips sample side down on drops of primary antibody in PBST with 0.4% BSA on parafilm. After an hour incubation, coverslips were washed as before and then incubated in secondary antibody in the dark. After an hour incubation, coverslips were again washed and

then placed on slides in mounting media with DAPI integrated. Slides were placed in 4°C to cure for at least 24 hours. Imaging was done with a ZEISS LSM 800 confocal microscope and images were prepared using the Zen Blue software.

Results

Characteristic	No pain, N = 12[1]	NSAID-resistant pain, N = 19[1]	NSAID-responsive pain, N = 5[1]	p-value[2]
Collection duration (hrs)	12.34 (10.50, 12.59)	11.26 (9.18, 12.04)	11.62 (10.92, 12.12)	0.2
Apprx Volume (mL)	8 (3, 10)	12 (8, 19)	11 (6, 15)	0.051
Weight (g)	7.4 (4.8, 8.8)	10.1 (8.6, 14.0)	7.0 (4.6, 10.4)	0.2
Missing	3	13	0	
Rate (mL/hr)	0.66 (0.25, 0.80)	1.36 (0.75, 1.67)	0.95 (0.54, 1.13)	0.024
Rate (g/hr)	0.54 (0.46, 0.69)	1.15 (0.93, 1.42)	0.60 (0.38, 0.78)	0.043
Missing	3	13	0	
Painkillers used during collection	1 (8.3%)	8 (42%)	2 (40%)	0.12
Worst pain during collection (0-100)	0 (0, 0)	50 (15, 62)	60 (29, 71)	<0.001
Average pain during collection (0-100)	0 (0, 0)	40 (0, 48)	22 (20, 50)	<0.001
Periods in the last 3 months				0.4
hormone-induced	4 (36%)	5 (26%)	0 (0%)	
natural	7 (64%)	14 (74%)	5 (100%)	
Missing	1	0	0	

[1] Median (IQR); n (%)
[2] Kruskal-Wallis rank sum test; Fisher's exact test

Table 6

Individuals without pain have lighter menstrual flow

The cohort consisted of 25 individuals with no significant differences in height or weight. The median age was 21 years and participants were of diverse ethnicities. We stratified participants based on menstrual pain and responsiveness to NSAIDs. We first focused on establishing if there were any differences in menstrual flow. We found differences in menstrual flow rate by pain group, as well as pain experienced during the menses collection (Table 6). Participants did not differ by pain group in their duration of menses collection, painkiller use, or hormone use (Table 6). Considerable variation exists in menstrual flow, both between individuals and between cycles within the same individual. Among the paired samples, we observed a correlation of 0.35 ($p = 0.3$) between first and second collection volumes normalized by collection duration, indicating that an individual's menstrual flow rate for one period does not predict another period's flow

rate. Previous studies on menstrual flow have focused on blood loss and have found that while there is great variability, most of the variation is seen in people with very heavy periods[77], that people generally do not fluctuate between very light periods and very heavy periods[79], and that menstrual flow per period tends to fit a uniform distribution[80]. In Figure 3-1, we observe that no pain participants had a lower median flow rate, measured as g/hr, than the NSAID-resistant pain participants ($p = 0.01$). The difference between NSAID-responsive pain and NSAID-resistant pain is not statistically significant, and there is no difference between no pain and NSAID-responsive pain.

Previous research has identified correlations between duration of menstrual flow and likelihood of dysmenorrhea, with a particular relationship between the presence of clots in the menstrual effluent and dysmenorrhea. Our findings demonstrate that individuals with NSAID-resistant pain have a higher flow rate of menstrual effluent as compared to no pain controls, independent of the duration of their periods.

Individuals without pain are less likely to have successful MenSC isolation

We next explored the efficiency of MenSC isolation and successful cell line generation across the different groups. In addition to decreased menstrual output, persons who do not experience pelvic pain donated samples that were less likely to yield successful MenSC cultures (Figure 3-2). While volume of sample donation was certainly correlated with success of MenSC isolation, persons with NSAID-resistant pain had successful MenSC isolation even at low donation volumes. Every NSAID-resistant pain sample had a successful MenSC isolation (100%). NSAID-responsive samples yielded MenSCs 80% of the time, likely associated with the higher volumes donated as compared to the no pain participants. In contrast, two thirds of the no pain samples had successful MenSC cell line generation. This difference between groups is

statistically significant (Fisher's exact test, $p = 0.03$). In Figure 3-2, we show that unsuccessful MenSC isolation is associated with lower sample volumes and that successful MenSC isolation in NSAID-resistant samples have greater variation in sample volume. These findings suggest that people who do not experience menstrual pain may have a lower abundance of MenSCs in their menstrual effluent in addition to decreased volume.

NSAID-resistance is independent of COX-2 expression

Cyclooxygenase-2 (COX-2) is the transiently expressed prostaglandin synthase that is both responsible for the production of prostaglandins that induce menstrual cramping and is the target of NSAIDs. We next wanted to test the hypothesis that people who report NSAID-resistant pain have greater expression of COX-2, which could render it difficult to inhibit. We find variable expression of COX-2 regardless of pain status, with the highest expression among people with NSAID-resistance (Figure 3-3). However, expression of COX-2 is also observed among the no pain controls, suggesting that COX-2 expression levels do not correlate with the presence or absence of menstrual pain or its responsiveness to NSAIDs.

MenSCs from people with no pain express less NANOG

Expression of stem cell markers in MenSCs have been previously reported, although consensus on which stem cell markers are consistently expressed has not been reached. We next explored the expression of stem cell markers in MenSCs to define their stem cell identity. Three genes have been identified as essential to maintaining pluripotency in embryonic stem cells: NANOG, SOX-2 and OCT-4[93] and these genes have been reported to be expressed in MenSCs[94]. While RT-PCR for OCT-4 did not identify any expression in any of the MenSCs (Figure 3-4), SOX-2 is expressed at low levels inconsistently across MenSC cell lines. Interestingly, NANOG expression is correlated with pain status, with those with no pain showing decreased NANOG

expression (Figure 3-4). Our findings suggest that MenSCs express markers of stemness that correlate with pain status.

Starvation stress downregulates mTOR C2 in NSAID-resistant pain

Given our findings of differences in menstrual flow rate by pain status, as well as success of MenSC generation, we wanted to explore if there would be differences in growth signaling in response to environmental stress. Under normal culturing conditions, MenSCs appear morphologically like fibroblasts with extensive actin cytoskeleton filling the gaps between cells (Figure 3-4B and 3-4J). After nutrient deprivation, the cells remodel, becoming more compact and rounded, and exhibit decreased actin staining (Figure 3-4F and 3-4N). We have noted that MenSCs are resilient cells and can be brought back to confluence after extended periods of starvation stress (data not shown) suggesting that they are sensitive to nutrient availability. One of the main pathways that regulate cellular response to starvation is the mTOR signaling pathway, a nutrient sensing signal that alters cellular processes in response to loss of glucose and key amino acids[81]. Under conditions of nutrient availability, p-mTOR S2481, a marker of mTOR complex 2 activation[95], is primarily located in the nucleus in no pain control cells (Figure 3-4C). A similar pattern is present in NSAID-resistant pain cells, but at much higher levels of expression (Figure 3-4K). In contrast, p-mTOR S2481 propagates throughout the cytoplasm in response to starvation stress in the no pain controls (Figure 3-4G) as compared to its occasional nuclear localization under normal growth conditions (Figure 3-4C). While similar localization patterns are seen in MenSCs from those with NSAID-resistant pain, p-mTOR S2481 is downregulated in response to starvation stress (Figure 3-4O). Our findings demonstrate that the response to starvation stress by MenSCs is different depending on the pain status of individuals.

Discussion

We have isolated MenSCs from participants with no menstrual pain, with NSAID-resistant menstrual pain, and with NSAID-responsive menstrual pain and found differences by pain phenotypes in growth attributes. We first describe a difference in the menstrual flow rate on the first or second day of the period independent of period duration, with a reduced flow found in those with no pain. We then find that a decreased volume of sample in the no pain group is associated with a failure to isolate MenSCs while no such difference is seen in the NSAID-resistant pain group. This suggests that people who do not experience menstrual pain may have a lower abundance of MenSCs in their menstrual effluent. We find variable expression of COX-2 regardless of pain status, suggesting that COX-2 expression levels do not correlate with the presence or absence of menstrual pain or its responsiveness to NSAIDs. In contrast, we see a marked difference in NANOG expression by pain status. Finally, we demonstrate that the response to starvation stress by MenSCs is different depending on the pain status of individuals. MenSCs from no pain controls upregulate mTOR C2 in response to starvation stress while those from NSAID-resistant pain downregulate.

We have found that there is a significant relationship between volume of menses and the presence of pain, and we have demonstrated differential signaling of mTORC2 in MenSCs derived from individuals with NSAID-resistant pain. It is possible that menstrual pain is a natural consequence of increased endometrial growth. In the setting of a larger amount of tissue resulting from increased growth, we would predict larger amounts of the substrate required for the conversion of arachidonic acid to prostaglandins. Additional studies looking at the relationship between growth signaling pathways and prostaglandin biosynthesis and COX-2 activity could discover mechanisms that control menstrual volume or MenSC abundance.

MenSCs are stem cells and we have demonstrated that they express stem cell markers. We find differential expression of NANOG, a homeobox gene that is best known for its function in suppressing cell differentiation pathways and maintaining pluripotency. However, NANOG is also connected to COX-2. NANOG is known to co-express with COX-2[96] and inhibition of COX-2 results in decreased NANOG expression[97]. Furthermore, treatment with PGE2 increases NANOG mRNA expression and protein levels[98]. Our findings further corroborate this link between COX-2 and NANOG, given that we find increased expression of NANOG in MenSCs from people with dysmenorrhea and the greatest expression in those with NSAID-resistant dysmenorrhea. Further research into the expression of NANOG in the context of endometriosis-associated pain may identify more effective therapeutic targets than COX-2.

Limitations of this study include our small sample size, especially within the NSAID-responsive group, and the unknown endometriosis status of all participants. We cannot rule out that the no pain controls could have asymptomatic endometriosis, but we do know that they do not have endometriosis-associated pain. Future research with participants of known endometriosis status could determine whether these results are endometriosis-specific or generalizable to all dysmenorrhea.

Figure 3-1

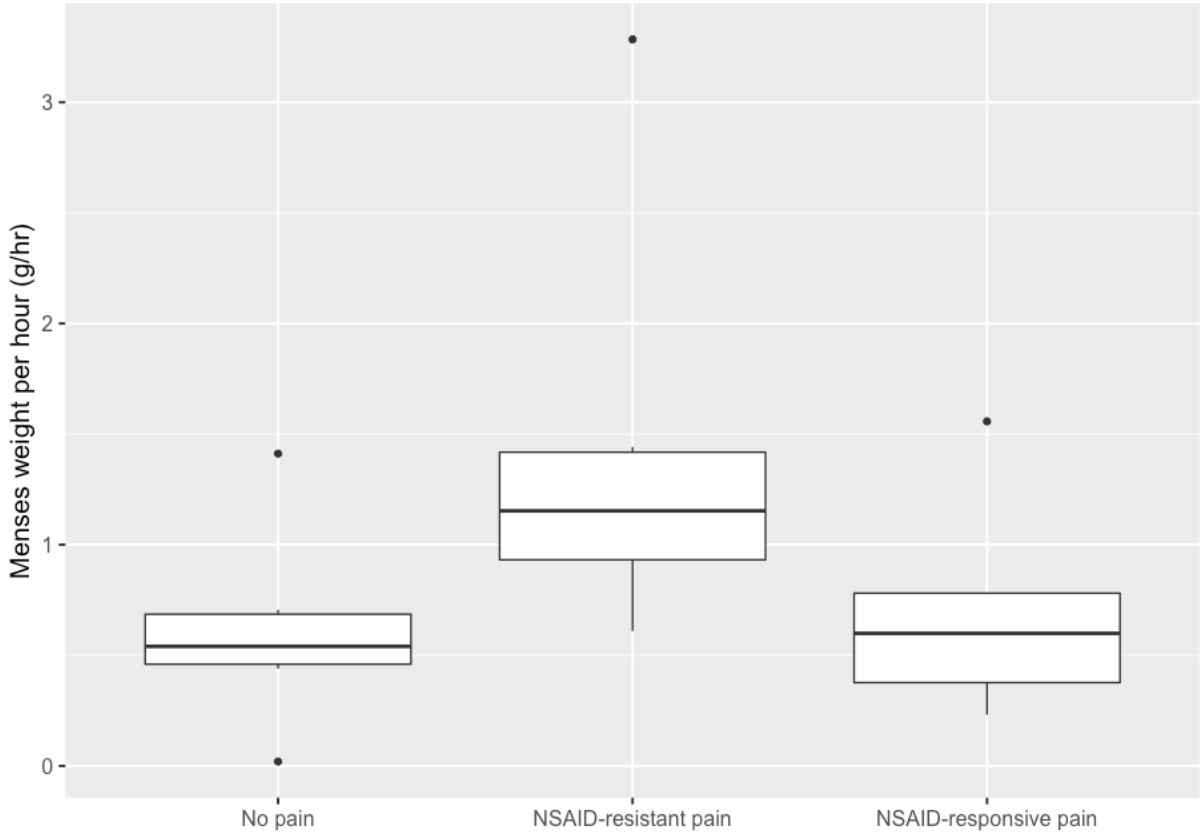

Figure 3-1: Individuals with NSAID-resistant pain have a higher menstrual flow rate

On the y-axis is the weight of the menses sample collected by individuals measured in grams and normalized by the number of hours spent collecting the sample. The mid-line is the median and the upper and lower extents of the boxes represent the interquartile range.

Figure 3-2

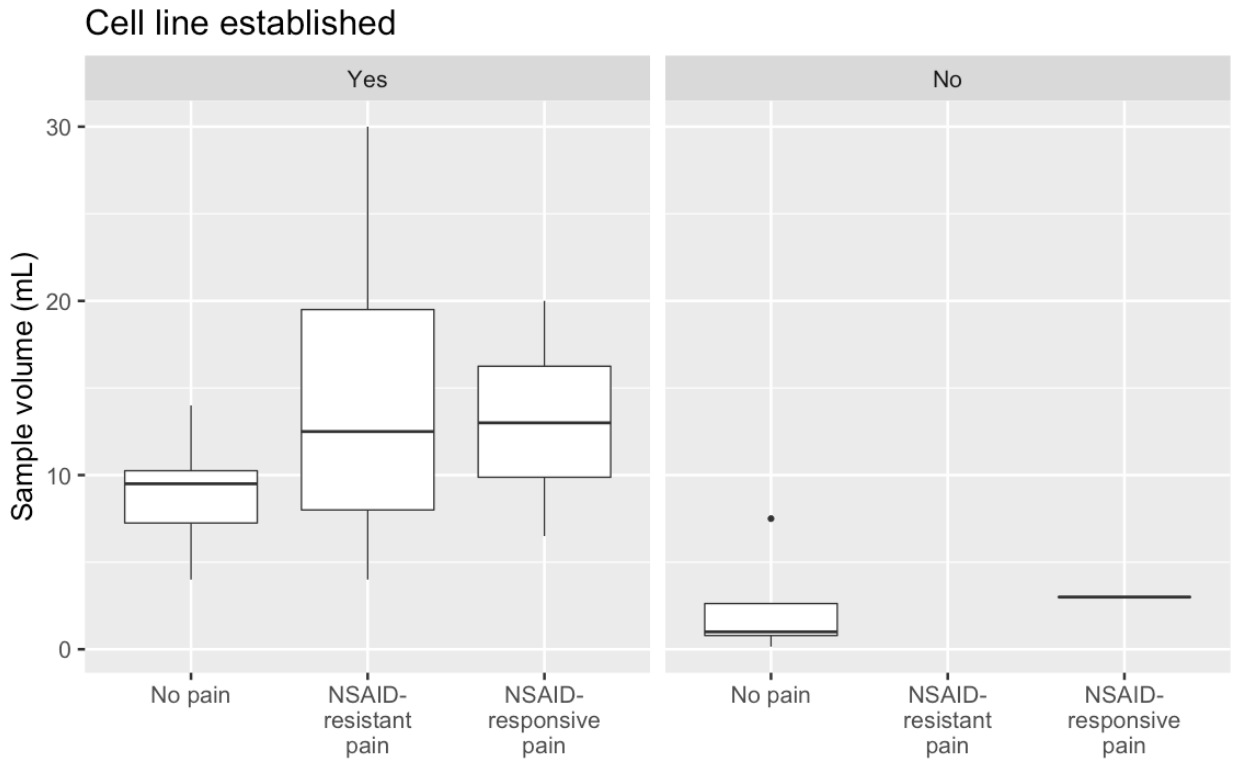

Figure 3-2: Individuals with no pain have lower abundance of MenSCs

On the y-axis is the estimated volume of the menses sample measure in mL. The left facet represents those samples that yielded a cell line of MenSCs and the right facet represents those samples that yielded no MenSCs. Sample volume is correlated with isolation success, but those with NSAID-resistant pain had successful cell isolation even at low volumes.

Figure 3-3

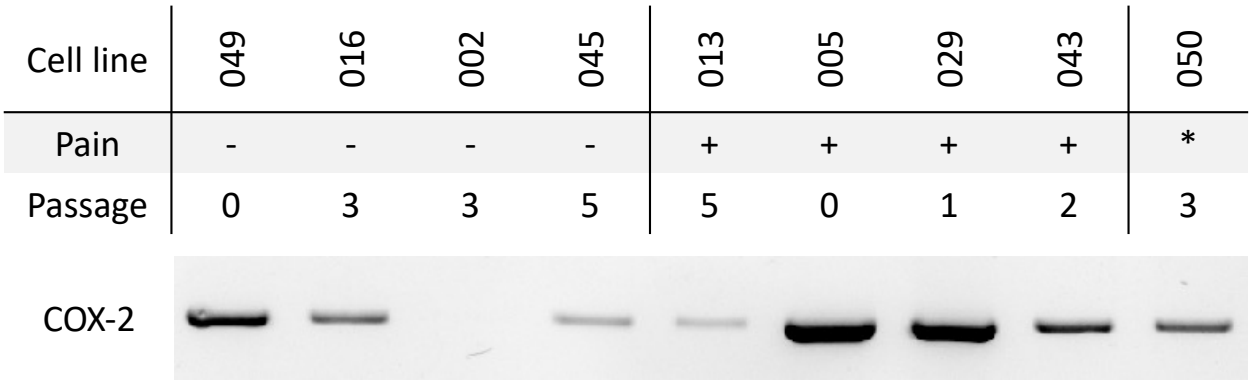

Figure 3-3: COX-2 expression does not correlate with pain status

RT-PCR for COX-2. All cell lines are MenSCs. Pain group is represented as (-) for no pain controls, (+) for NSAID-resistant pain, and (*) for NSAID-responsive pain.

Figure 3-4

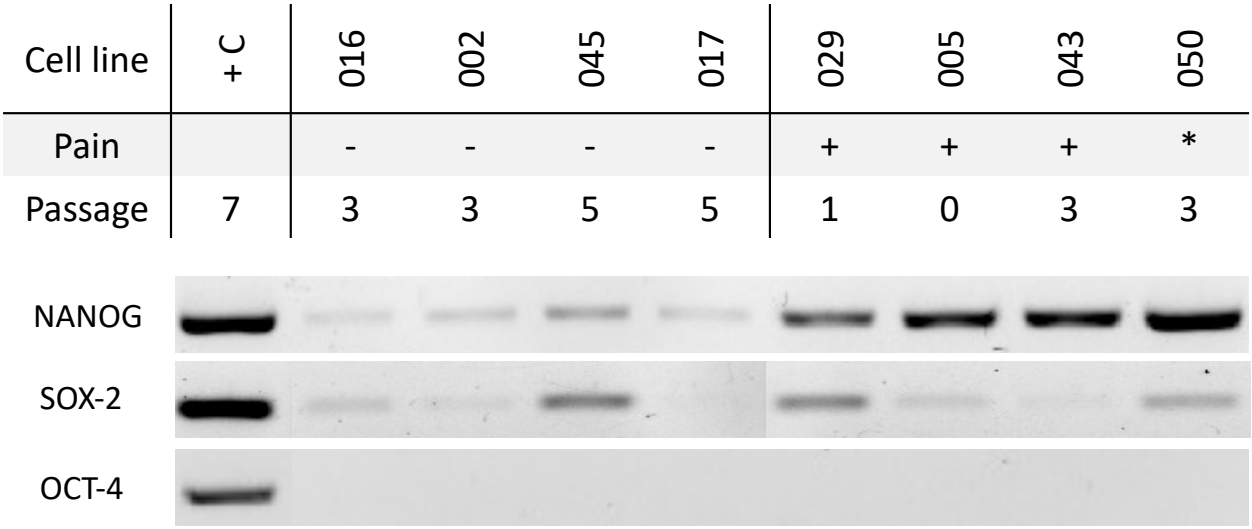

Figure 3-4: NANOG expression is differentially expressed by pain status

RT-PCR for NANOG, SOX-2, and OCT-4. Positive control is H9 embryonic stem cells. Remaining cell lines are MenScs. Pain group is represented as (-) for no pain controls, (+) for NSAID-resistant pain, and (*) for NSAID-responsive pain.

Figure 3-5

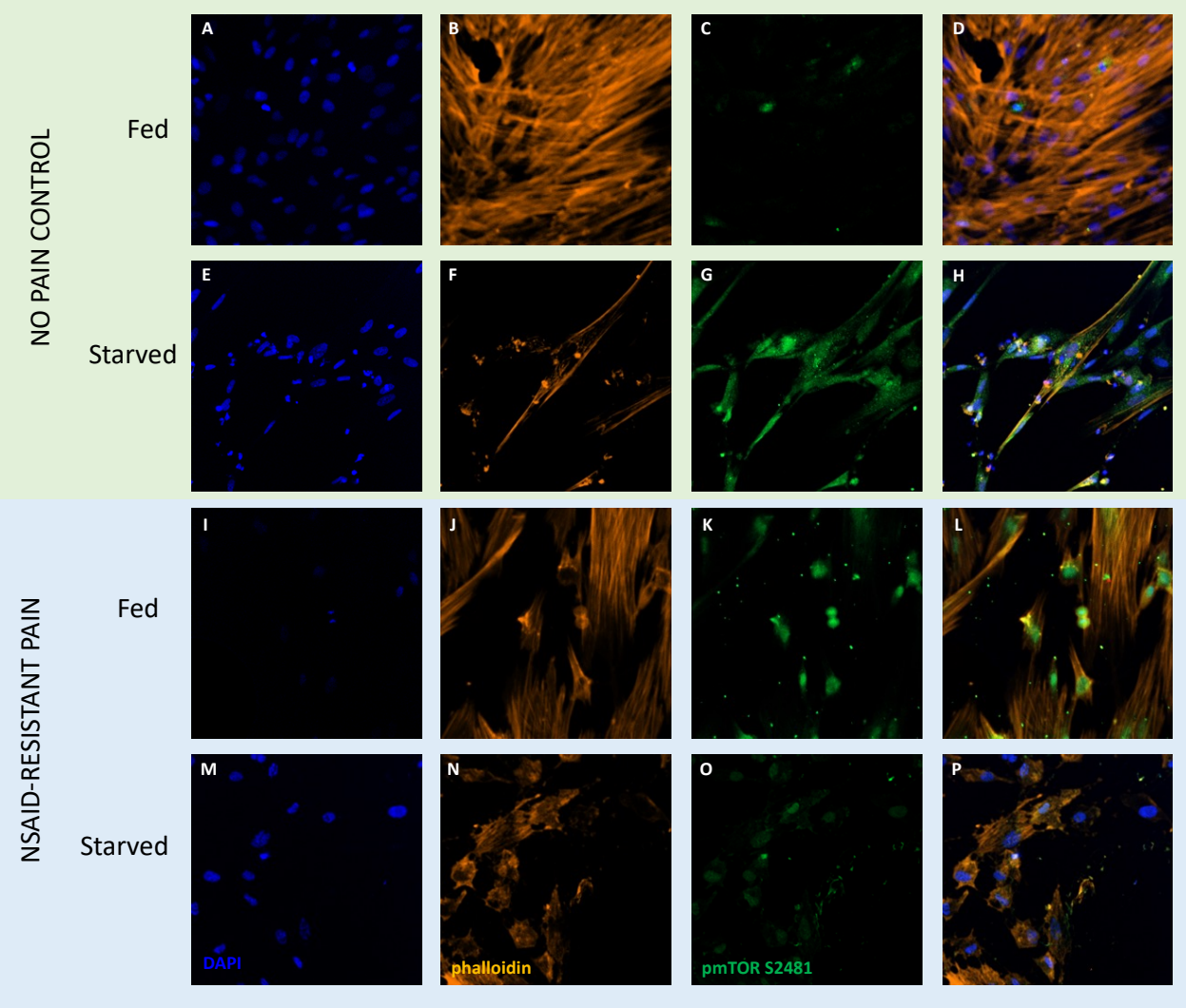

Figure 3-5: mTORC2 is dysregulated in MenSCs from individuals with NSAID-resistant pain under conditions of nutrient starvation

Images were taken at 20X. The blue channel is DAPI; the orange channel is phalloidin; the green channel is pmTOR S2481 (Cell Signaling #2974).

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