MANUSCRIPT

Endometriosis: A Mechanistically-Grounded Hypothesis for miRNA-Mediated Pre-EMT Apoptotic Regulation as a Therapeutic Strategy

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Endometriosis is a long-lasting reproductive health condition caused by the abnormal development of endometrial-like tissue where it does not belong. According to worldwide estimates, 10% of all women of childbearing age are affected by endometriosis. The current treatments for endometriosis are limited due to the tendency of the disease to recur and the systemic side effects of existing hormonal therapies (including the use of the combined hormonal contraceptive pill). Recent studies have suggested that the development of endometriosis may be related to the dysregulation of apoptosis, improper epithelial-mesenchymal transition (commonly referred to as EMT), and changes in miRNA expression patterns.

This paper proposes a mechanistically grounded therapeutic treatment strategy for endometriosis based on the proposal that inducing programmed cell death (apoptosis) in endometrial cells via miRNA-mediated mechanisms with specificity to the time just prior to activation of EMT may limit ectopic implantation during retrograde menstruation. A series of experimental phases, which are listed within this paper, is outlined to enable experimental evaluation of this hypothesis. By targeting the upstream survival signaling pathways, this approach will provide a potential non-hormonal therapeutic strategy for endometriosis.

miRNA regulation

Apoptosis

Epithelial-Mesenchymal Transition (EMT)

Endometriosis

INTRODUCTION

Endometriosis is a long-standing disorder that impacts at least one in ten reproductive-age women around the world. Endometriosis is characterized by the presence of endometrial-like tissue outside the uterine cavity. Moreover, women suffering from endometriosis experience additional conditions such as: dysmenorrhea (painful periods), dyspareunia (painful intercourse), infertility, and decrease in quality of life. Even though there is no shortage of women across the world who have been diagnosed with endometriosis and affected in some way, treatment options available to these women can vary based on their specific individual needs and preferences. Often times, a surgical excision takes place but recurrence of the lesion is a common occurrence. Hormonal therapies may have systemic effects due to a woman's body mass index (BMI), and are not recommended for some women; therefore, novel treatment strategies are needed that target the underlying biological mechanisms of endometriosis rather than merely treating its symptoms.

The biological mechanisms that contribute to endometriosis are multiple. In agreement, retrograde menstruation is frequently cited as an environmental factor contributing to the development of endometriosis; however, since so many women experience retrograde menstruation and do not develop endometriosis, it can be inferred that other environmental and molecular mechanisms may also be involved in increasing the severity of a woman with endometriosis. Recent studies have suggested that at least three different processes appear to promote the progression of endometriosis: decreased apoptosis of the endometrial tissue, aberrant expression of the epithelial-mesenchymal transition (EMT), and dysregulation of the expression of microRNAs (miRNAs).

During the monthly menstrual cycle, apoptosis plays a key role in the removal of abnormal or non-usable cells from the lining of the uterus (the endometrium). Endometriosis results from the presence of abnormally growing cells that should otherwise go through apoptosis (programmed cell death) but were able to avoid it and continue growing. Furthermore, dysregulated epithelial mesenchymal transition (EMT) increases the ability of the cells to migrate and invade into new sites, resulting in the implantation of these cells at ectopic locations (locations that are not normal for uterine tissue). MiRNAs (microRNAs) are posttranscriptional regulators of gene expression and were recently recognized as significant regulators of both apoptotic signaling pathways and the EMT. Several specific miRNAs (such as miR-195, miR-503, miR-141-3p, and miR-205-5p denoted in the references below) have been shown to have altered levels of expression when comparing endometriotic tissue to non-endometriotic (healthy) tissue, and have also been implicated in the progression of endometriosis.

The above suggests that developing therapeutic agents directed at modulating apoptotic and/or EMT-related miRNA expression may serve as potential therapies for preventing ectopic implantation and development of lesions associated with endometriosis.

THE HYPOTHESIS

This article hypothesizes that the continued presence and dissemination of endometrial cells from endometriosis results from failure of susceptible cells to undergo apoptosis before activating migratory pathways through epithelial-mesenchymal transition (EMT) pathways. The article proposes that specific miRNAs may restore programmed cell death prior to EMT activation. By this method, the number of viable endometrial cells available to implant ectopically during retrograde menstruation will be decreased.

During normal physiological conditions, the monthly cycles of blood loss lead to cyclical increases and decreases of the hormone progesterone; these changes cause the lining of the uterus to grow and shed, which in turn causes the degeneration of matrix material between cell walls (through the activity of matrix metalloproteinases), and therefore the tissues of the uterus. Although apoptotic elimination of senescent or damaged cells typically occurs during these processes, women who are affected by endometriosis' inability to carry out normal cell death could contribute to the continued persistence of aberrant endometrial stromal cells beyond their expected lifetime. The activity of miRNAs is thought to be crucial to the regulation of these cellular processes. MiR-195 and miR-503 are involved with stimulating apoptotic pathways and suppressing cellular reproduction, while miR-141-3p and miR-205-5p play a role in maintaining an epithelial phenotype and controlling the migration-associated pathways (epithelial-mesenchymal transition—EMT). As a result, altered expression of all four of these miRNAs will lead to decreased apoptosis as well as enhance potential for migration.

According to the central hypothesis, there will be enhanced susceptibility to apoptosis in endometrial cells ready for shedding by a selective increase of expression of pro-apoptotic miRNAs along with resumption of normal modulating miRNA expression of EMT prior to the activation of EMT. Inducing cell death before the full activation of migratory pathways means that there will be fewer viable cells available for retrograde dissemination and clinical implantation. This approach should therefore result in decreased lesion development while still maintaining normal function of the uterus and avoiding the systemic effects that accompany hormonal therapies.

EVOLUTION OF HYPOTHESIS

Three independent observations about dysregulated epithelial-mesenchymal transition (EMT), impaired endometrial cell apoptosis and altered microRNA (miRNA) expression in the literature led to this hypothesis being developed as a result of their convergence. The normal menstrual cycle is characterized by cyclical increases and decreases in levels of progesterone,

which regulate the remodeling and shedding of the endometrium. These events also involve the degradation of the extracellular matrix by matrix metalloproteinases (MMP-2 and MMP-9), as well as the removal of senescent or damaged cells via apoptosis, thus allowing for an effective means of removing excess endometrial tissue during a menstrual period. However, studies have shown that when endometrial cells are obtained from women with endometriosis, they demonstrate increased resistance to apoptosis, meaning that some endometrial cells that are typically programmed to undergo cell death will not do so in women with endometriosis.

Concurrently, evidence exists pointing to the role of EMT in providing the increased migratory and invasive characteristics of endometrial cells associated with endometriosis. Loss of cell-cell adhesion, which is typical of epithelial cells transitioning to a mesenchymal phenotype during the EMT process, allows for increased mobility and the ability for endometrial cells to implant in ectopic sites. The elevated expression of markers associated with EMT in endometrial lesions provides evidence that this transition may play a critical role in the establishment of endometriotic lesions following retrograde menstruation.

MicroRNAs potentially link these pathological processes by causing alteration in apoptosis and epithelial-mesenchymal transition (EMT) cellular signaling pathways through multiple miRNAs related to endometriosis. Specifically, miR-195 and miR-503 involve pro-apoptotic activity with a decrease in cellular proliferation; whereas, miR-141-3p and miR-205-5p maintain epithelial characteristics through regulating EMT-associated signaling. The aberration of these miRNAs expression can promote cell viability through enhancing cell migratory traits at the same time.

These findings have led to the formulation hypothesis detailed below. It may be possible to intercept the disease prior to the time of established lesions in uterine tissue by restoring apoptosis well before there is an activation of EMT (in other words, preventing an increase in viable endometrial cells during retrograde menstrual flow). By selectively increasing pro-apoptotic miRNAs immediately before the cellular signal (i.e., time of activation) for EMT, the pool of cells that can become ejected from the uterine cavity will be substantially decreased. Utilizing this type of temporal intervention will hopefully minimize (or eliminate) ectopic implantation while maintaining regular uterine physiological function.

The development of this hypothesis is based on the understanding that EMT and resistance to apoptosis can be thought of as two parts of a process that allows endometrial cells to stay alive and move from one place to another. Investigating the mechanisms by which miRNAs regulate both pathways provides a valid, scientifically based means for intervening in the onset of disease earlier than is currently possible using existing therapies.

HYPOTHESIS TESTING

Phase I: Assessing the Expression of miRNAs and the Phenotypes of Endometrial Tissue

Endometrial tissues will be obtained via biopsy from 40 women with endometriosis and 20 matched women without endometriosis who are undergoing other gynecological surgery. Biopsies will be performed during the proliferative phase of the menstrual cycle to reduce variability due to hormones. Biopsy tissue will be digested by enzymatic digestion and various fractions (stromal and epithelium cell fractions) will be isolated by differential centrifugation. These fractions will then be characterized using flow cytometry and immunostaining.

To evaluate if the miRNAs have differential levels of expression in the two conditions, we will measure apoptosis (caspase 3), proliferation (Ki67), and the activation of the EMT (vimentin and N-cadherin). The expression of the candidate miRNAs (miR-195, miR-503, miR-2861, miR-141-3p, and miR-205-5p) will be assessed by quantitative polymerase chain reaction (qPCR) as well as validated by small RNA sequencing.

Phase II: The Modulation of miRNA Activity

After isolating endometrial cells, synthetic miRNAs (mimics and inhibitors) will be transfected into the cells by the optimized use of lipids or electroporation. After transfection, apoptosis will be quantified using Annexin V/propidium iodide staining, TUNEL assays, and caspase 3 activity assays. A progesterone-withdrawal culture system will be used as a model for studying the physiological timing of menstruation (PM). The PM model will be used to determine if the induction of apoptosis via miRNA modulation and the activation of EMT-related pathways are temporally dissociated. Molecular assays and wound healing assays will be used to assess Notch1 signaling, MMP-2/MMP-9 expression, and cellular migration.

The goal of this phase is to determine if targeted modulation of miRNA causes apoptosis in a selective manner prior to the activation of EMT, and to decrease the number of viable cells with the ability to disseminate. To quantify EMT (epithelial-mesenchymal transition), we will quantify a panel of established epithelial & mesenchymal markers. EMT (epithelial-mesenchymal transition) will be quantified by a significant decrease in E-cadherin, with a concomitant increase in mesenchymal markers (e.g., vimentin and N-cadherin) and an increase in activation of the notch 1 signaling pathway and matrix metalloproteinases (MMP-2, MMP-9). These changes will be quantified by qPCR, western blotting and immunofluorescence. In experimental studies, EMT activation will occur when there is a consistent pattern of decreased E-cadherin and increased vimentin, N-cadherin, and notch 1 signaling relative to control. This operational definition will allow us to determine whether miRNA mediated apoptosis occurs prior to the onset of EMT associated transformation and migration.

Phase III: Assessment of Immune Interaction and Tissue Level

The downstream biological activity of miRNA-modulated endometrial cells, combined with macrophages derived from peripheral blood monocytes, will be assessed by co-culture of

treated endometrial cells with macrophages in ex vivo uterine tissue scaffolds. Cytokine production, macrophage infiltration, phagocytosis, and inflammation evaluation will be completed using ELISA, live-cell imaging, and flow cytometry. Dose-response studies using miRNA modulation will be performed to identify the optimal concentration of miRNA modifiers for inducing targeted apoptosis while preserving surrounding healthy endometrial tissue.

Computational modelling will be completed to integrate miRNA activity, progesterone signaling, and EMT kinetics in order to predict the timing and potential systemic effects of the proposed therapy. Statistical significance for all experiments will be established by performing all experiments in triplicate and defining statistical significance at $p < 0.05$.

IMPLICATIONS

If this study is confirmed, it will set a new treatment approach for endometriosis unlike anything seen before. Current therapies deal only with symptom management or surgically removing already formed lesions, while this new approach proposes to help prevent new lesions from developing by modifying the signaling pathways in our cells before the disease has progressed to the stage of lesion (damage) formation. If we are able to increase the chance that an endometrial cell will die (unlike what happens normally) prior to the initiation of a specific type of cellular change (epithelial-mesenchymal transition or EMT), there will be fewer endometrial cells that can grow back through retrograde menstruation. This will increase the likelihood that women maintain normal reproductive function and decrease the chances that their cells will implant abnormally or form lesions.

There is evidence that supports this theory, that endometriotic tissue resists cell death and that their cells have increased EMT activity and that several microRNAs are known to modulate these pathways. Therefore, if we can return the expression of the microRNAs to normal levels, this will restore the normal balance between survival and death in cells that have become out of balance in endometriosis. There are multiple potential issues that need to be resolved before there is the possibility for clinical use of these therapies. First, we will need to create appropriate delivery systems that can target specific tissues and avoid affecting other tissues.

Second, we must carefully consider any off-target effects (unintended cell death in nearby healthy cells) of the therapy prior to using this treatment. Third, the efficacy of the therapeutic approach may be time-sensitive based on the pattern of hormonal fluctuation and windows of susceptibility of the cells for undergoing EMT. Regardless, the proposed hypothesis provides a mechanistically and scientifically sound framework for testing a non-hormonal approach to preventing establishment of endometriosis through the temporally controlled and miRNA-mediated induction of apoptosis.

There is currently little direct evidence linking all potential miRNAs with endometriosis; however, studies of various other diseases (that have been examined) have demonstrated that

specific miRNAs are involved in some aspect of regulating apoptosis, cell proliferation, migration, and epithelial-mesenchymal transition. In addition, these same processes have all been shown to be important in the pathophysiology of endometriosis. Based on the previously mentioned observations, as well as those of other related biological systems, it can be hypothesized that there may be similar mechanisms regulating these cellular events within endometrial tissue and therefore attempt to integrate the miRNA literature, as well as literature specifically focusing on the relationship between miRNAs and endometriosis, to identify pathways that may be useful clinically in this neglected area of research.

CONCLUSION

Endometriosis continues to be a common, difficult to manage condition that often doesn't completely improve with current therapies. In this study, we propose that failure of apoptotic processes to occur prior to initiating an epithelial-to-mesenchymal transition (EMT) increases the survival and dissemination of viable endometrial cells when they enter into the peritoneal cavity through retrograde menstruation. Furthermore, we suggest that by specifically modulating certain miRNAs, we may be able to induce apoptotic cell death within the critical period before epithelial-mesenchymal transition occurs and thus reduce the likelihood of ectopic implantation and/or formation of lesions. Our proposed experimental design offers a framework to test this hypothesis and could provide a foundation for developing a non-hormonal therapeutic option for women suffering from endometriosis.

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